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January 2, 1922.

NOTICE TO AUTHORS AND COMMUNICATORS.

The Council have had under consideration the rapid increase of the Society's expenditure on publications. In view of the necessity for economy, authors of papers are urgently requested to see that their communications are put in as concise a form as possible. Delay in decisions regarding publication, as well as subsequent trouble to authors, is often caused by diffuseness or prolixity. MSS. must be type-written or at least written in a legible hand, and properly prepared as copy for press. Type-written transcript should in all cases be carefully revised by the author before being presented. It is desirable that authors should retain copies of their MSS. for reference.

Every paper must be accompanied by a summary not exceeding 300 words in length.

Authors are requested to refer to a Memorandum on Mathematical Notation published in these 'Proceedings,' 1909, Series A, vol. 82, p. 14, and to adhere to the suggestions therein contained, so far as possible.

Authors are further requested to send in all drawings, diagrams or other illustrations in a state suitable for direct photographic reproduction. They should be drawn on a large scale in Indian ink on a smooth white surface, with temporary lettering in pencil. Great care should be exercised in selecting only those that are essential. Where the illustrations are numerous, much time would be saved if the authors would indicate in advance those which, if a reduction of their number is found to be required, might be omitted with least inconvenience.

"It shall be the duty of each Fellow or Foreign Member to satisfy himself that any letter, report or other paper which he may communicate, is suitable to be read before the Society."—Statute 64, Cap. xii.

NOTICES TO FELLOWS OF THE ROYAL SOCIETY.

The Council have directed that the Minutes of the Meetings of the Society shall be sent out as an inset in the 'Proceedings,' separately paged. In future they will not be republished in the 'Year-Book.'

The 'Proceedings' are sent in the ordinary course by post to those Fellows of the Society who have applied for them. On application to Messrs. Harrison and Sons, Ltd., St. Martin's Lane, London, W.C. 2, these will be bound in volumes, in cloth, for 5s., or the cases for binding may be purchased, price 2s. 3d.

The 'Philosophical Transactions' are now issued in the form of separate Papers, and are delivered post free, immediately on publication, to those Fellows who desire to have them in that form. On application to Messrs. Harrison and Sons, Ltd., St. Martin's Lane, London, W.C. 2, these issues will be retained, and supplied in volume form in cloth binding.

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PROCEEDINGS OF
THE ROYAL SOCIETY.

Section B.—Biological Sciences.

Address of the President, Prof. C. S. Sherrington, at the Anniversary Meeting, November 30, 1921.

Since the last Anniversary Meeting the roll of the Society has lost by death fifteen Fellows and one Foreign Member :

Sir William Abney.
Mr. Spencer Pickering.
Dr. A. Muirhead.
Sir Lazarus Fletcher.
Prof. W. Odling.
Prof. L. C. Miall.
Prof. R. B. Clifton.
Lord Moulton.
Prof. A. W. Reinold.
Prof. E. J. Mills.
Colonel J. Herschel.
Mr. G. W. Walker.
Dr. H. Woodward.
The Earl of Ducie.
Dr. F. A. Bainbridge.

On the Foreign List

Prof. G. Lippmann.

The Anniversary Meeting affords appropriate opportunity for some spoken reference to them.

The earliest loss was that of William de Wiveleslie Abney, a Fellow of the Society for upwards of forty years. Much of his scientific work may be summarised as being the establishment, by experiment, of photography as a science. With Sir William Abney photography was not merely a means but in itself a scientific end. The building of the image both in the wet and in the dry plate were successfully studied by him. He was a pioneer in the photography of the infra-red region of the spectrum. He suggested more than forty years ago the charging of carbons with calcium salts to enhance the arc-light beam, the flame arcs of to-day. Later he passed, so to say, from the photographic plate to the retina and investigated the relative visual intensity of different portions of the spectrum. As Advisor to the Board of Trade he obtained, partly in collaboration with the late W. Watson, data most valuably discriminating between various types of colour vision; he contributed accurate measurements of visual differences between the foveal and para-foveal regions of the retina. His measurements of the visual luminosity curve of the spectrum stand as classical data of reference. He is remembered in the Society as a man whose personality endeared him to everyone who knew him.

The death of SPENCER PERCIVAL UMFREVILLE PICKERING removed a chemist, who at the time of his election to the Society, was one of the most arduous and prolific of researchers. The main theme of his work was solution and hydrates. A man of original view he often collided rather than moved with the scientific trend of the time, but he spared himself no pains in the pursuit of observations. His association with the Society will be happily perpetuated by the bequest from him, to become a research fund bearing his name.

ALEXANDER MUIRHEAD, whose name is connected with the duplexing of submarine cables by the artificial line with distributed capacity, also contributed perseveringly to the practical establishment of electrical standards of capacity. Much of his work was accomplished against difficulties of health which would have disheartened any but a man of remarkable courage and resolution.

LAZARUS FLETCHER was for ten years Director of the Natural History Museum. Mathematically trained, his chief scientific interest lay in problems connected with the physics of crystals, though much of his time was given to the great National Collection of minerals of which he had charge for nearly thirty years. He devoted much patient and accurate research to the meteorites in that collection. His papers that are probably best known are those on the dilatation of crystals by heat, and on the Optical Indicatrix and transmission of light in crystals. In the latter of these he showed how the optical characters of crystals could be simply developed from the geometrical properties of an ellipsoid (which he called the Indicatrix) independently of any hypothesis as to the nature of the ether. His method has now been adopted by almost all teachers of the subject. Those who knew Sir Lazarus Fletcher are not likely to forget his simplicity of manner, his quiet humour and his unfailing consideration for others.

WILLIAM ODLING, for many years Professor of Chemistry at Oxford, died there this spring at the age of ninety-two, severing a link with the chemistry of the mid-Victorian time. It was under his Chairmanship of the Institute of Chemistry that that body was granted its Charter in 1885.

LOUIS COMPTON MIALL was a biologist; a naturalist in the old sense of the word. He did good and lasting zoological research. He was one of a group, few in number but strong in personality and influence, who laid the foundation of the existing University of Leeds. He was an enthusiastic educationalist, and appreciated highly the calling and the opportunities of the primary school-teacher; he helped that calling in many ways. He himself was a strikingly successful teacher. Those who knew him will recall how he studied teaching as an art, and loved it for its own sake.

Dying at Oxford a little later in the year than Prof. Odling, ROBERT BELLAMY CLIFTON had been Professor of Experimental Philosophy there from 1865 until 1917. His first duty for his Chair had been the superintendence of the erection of the laboratory, the Clarendon Laboratory, of which Sir Richard Glazebrook writes in his obituary notice of Clifton: "it was the first built in Europe for the special purpose of experimental instruction in Physics." The fittings and teaching apparatus were largely to Prof. Clifton's designs, and he gave much time and thought to their construction, perfecting and re-perfecting them in detail. So strict a custodian of them did he become that it was sometimes humorously said they had become too precious to be very accessible for their original purpose. However that may be, under his hospitality the laboratory he had erected gave a home to a great piece of experimentation in Prof. Boys' determination of the gravitation-constant. Clifton was a man of genial personality, of much conversational gift, shrewd and humorous, and of a nature full of kindly qualities.

WILLIAM REINOLD was Professor of Physics in the Royal Naval College. He had been Demonstrator under Clifton in the Clarendon Laboratory. It was during his long activity at the Royal Naval College, and as a teacher there, that his main scientific life-work was accomplished.

In March last died suddenly Lord MOULTON OF BANK. Not an actual investigator in Science, he was yet a very real servant to the cause of scientific progress in this country. He possessed remarkable power of acquisition of knowledge, seizing rapidly and broadly the lines of advance taken by knowledge. A facile expositor of scientific themes to a lay or semi-lay audience, and gifted with an enthusiasm that never failed, he promoted the public appreciation of scientific work. Foreseeing from the outset of the War the magnitude of the strain that it would involve, he had the courage to demand a mobilisation of scientific resources adequate to that strain. The country owed much to his insistence and unsparing effort. His was a virile persuasion. After the coming of the Armistice, he turned his energies and influence toward urging a more thorough liaison between science and the industry of the country.

EDMUND JAMES MILLS held the Young Chair of Technical Chemistry in the Glasgow and West of Scotland Technical College. His papers were numerous both on applied and theoretical chemistry, and not a few of them were contributed to this Society, the first of them now more than fifty years ago. Returning to London in later life, he was for many years a frequent attendant at the Society's meetings.

Colonel JOHN HERSCHEL, a son of Sir John Herschel, and at one time Deputy-Superintendent of the Great Trigonometrical Survey of India, had been a Fellow of the Society for fifty years. He was a spectroscopic observer of a solar eclipse as far back as 1868.

GABRIEL LIPPMANN, the eminent French physicist, died while at sea on his way from Canada home to Paris. He had been a Foreign Member of the Society for five and twenty years. His interest in physics lay largely in the philosophic aspect, though his name is most familiar in connection with the capillary electrometer and with colour photography. Lippmann's capillary electrometer became, so to say, a household tool in every physical laboratory, and likewise in many biological laboratories. In animal physiology it proved of unique service for the observations of the slight and fleeting electromotive reactions of isolated nerve and muscle. Until the advent of the string galvanometer it was the only instrument which could really cope with them.

Of Lippmann's process for the reproduction of colour by photography, our Foreign Secretary, Sir Arthur Schuster, who knew him from a time when they were fellow students together, kindly writes me as follows:—"Lippmann's work on colour photography well illustrates his great experimental skill. Independently of the late Lord Rayleigh, who, in 1887, had on theoretical grounds foreseen the possibility of the reproduction of natural colours by an interference method, Lippmann conceived the same idea; but the experimental difficulties were formidable. The method depends on establishing a periodic structure in a photographic film by the interference of the direct light and its reflexion from a metallic surface. It was necessary for the purpose that the films unlike those in ordinary use, should be transparent. The production of such films appeared for many years to be an insoluble problem, but ultimately the difficulty was overcome, and in 1901 Lippmann obtained his first success; but it was several years before he could secure the equality of sensitiveness throughout the visible spectrum which is essential if the natural colours are to appear with their correct values. The photographs obtained by Lippmann cannot be reproduced in print, but may be shown with brilliant effect by projection on a screen."

George Walker Walker had, following on a career of high promise at Cambridge, been successively Lecturer in Physics at Glasgow University, Superintendent of Eskdalemuir Observatory, Director of the new Magnetic Survey of the British Isles, and finally chief scientific worker at the Royal Naval Mining School, Portsmouth. It was during work in that latter capacity that his fatal illness began. He united in a remarkable degree mathematical attainment and inventive capacity. By his death physical science lost, sadly early, a finely accurate experimental exponent.

Henry Woodward, late Keeper of the Department of Geology of the Natural History Museum, was a distinguished paleontologist. His scientific reputation was especially as an authority on extinct representatives of the Crustacea. He was one of the founders, and for over fifty years editor, of the 'Geological Magazine.' His example and personal contact were a stimulus to many others, and the encouragement given by him to amateur workers was one of the features of his official career.

Francis Arthur Bainbridge died last month in early middle age. He had been elected a Fellow in 1919. Of delicate physique, constantly struggling against ill-health, he nevertheless accomplished, besides much routine teaching, a great deal of accurate research, some in pathology, more in physiology. He contributed to the differential recognition of the several types of paratyphoid bacilli, a matter at once of theoretical interest and great practical importance. His work in physiology opened with investigation of lymph formation, following on that of Bayliss and Starling. Then came work on urinary and salivary secretion, all of it characterised by great clearness of objective, and definiteness of plan. One of his best papers is one of his most recent. Its subject is the acceleration of the pulse, which muscular exercise constantly and so quickly induces. Bainbridge showed that the increased filling of the venous chamber of the heart, and the consequent increase of pressure in it, itself acts as a stimulus which excites through the nervous system the more frequent beating of the heart. He traced this control in part to depression of the vagus, partly to stimulation of the nerves which accelerate the heart. Bainbridge was an experimentalist of exceptional dexterity. Always cheerful, he seemed at his cheeriest when busiest in the laboratory.

Lord Ducie, whose decease fell latest in the year, had been a Fellow for nearly 67 years. Interested in Science, he was also greatly interested in secondary education. Latterly he had given his time and abilities chiefly to the countryside where he resided. By virtue of the date of his election to the Society, 1855, he had become its Senior Fellow.

We may note that the Seniority of Fellowship of the Society has now passed to one who has been a member of Council on many occasions, a Foreign Secretary, and Secretary, our sometime President, Sir Archibald Geikie, known among us also as the genial historian of the Royal Society Club.

It is little more than two years since the death of the late Lord Rayleigh, and this afternoon in Westminster Abbey there has been unveiled the tablet to his memory, given by subscribers from this Society and from the University of Cambridge, of which he was Chancellor. At the presentation ceremony the Society and the donors generally were represented by the Chairman of the Memorial Committee, Sir Joseph Thomson. The Society will feel it peculiarly appropriate that their representative on such an occasion should be one so closely associated with the late Lord Rayleigh in the Society, in the University which was their common *alma mater*, and in the domain of physical science itself. The recollection of the late Lord Rayleigh's personality is present with us all: to meet him was to receive the impression of true greatness. The legend on the mural tablet runs:—"*An unerring leader in the advancement of Natural Knowledge.*" To-day has seen the fulfilment of a fitting tribute, in a fitting resting-place, to a memory veneration for which the lapse of time will but intensify.

The Bakerian Lecture of the year was by Dr. T. M. Lowry and Mr. P. C. Austin on "Optical Rotatory Dispersion." The Croonian Lecture was by Dr. Henry Head. It had for its theme the disturbance of action in the nervous system due to the impairment of one part reacting on the function of another. Not unnoteworthy concerning the lecture is that, to push further the enquiries underlying it, the lecturer had subjected to surgical severance and restitching nerves of his own arm.

To Dr. Head the Society owes a most acceptable gift. The Society possessed no portrait of Lord Lister. Dr. Head, on learning this year that such was the case, offered to the Society a portrait of Lister, by Legros, in black and white, a portrait that had been given to Dr. Head by the poet Henley, in whose possession it long was,—Henley, the poet whose word-portrayal of Lister, under whom he was a patient, is extant in the famous sonnet familiar to us all. The gift was gratefully accepted by Council.

The Anniversary Meeting is naturally an occasion for retrospect; it is also one which invites some thought to the present. The present time has in it an element of considerable anxiety for those who regard the prosperity of Science. Although the recent past has, it is true, been not unfavourable.

I mentioned just now a university building, the earliest constructed for systematic experimental teaching in Physics, and that just 50 years ago. It is a satisfaction to note the multiplication of such laboratories since then. This year at the inauguration in London of the Institute of Physics Sir Joseph

Thomson remarked that now, in contrast against the early years of the Cavendish Laboratory, the study of Physics, as regards the numbers to whom it gives opening for a livelihood, constitutes in fact a profession of its own. The same can be said of the Science of Chemistry, and of the Biological sciences. Cultivation of science has been a feature of the country's progress. This has in part been adjunct to the movement for the foundation of new Universities. The number of the English Universities has doubled in the last quarter of a century. The new Universities have shown admirable energy in their departments of science. Following in the tradition of the best of the older Universities they have, in instance after instance, made their laboratories places of research. Only last year the Council of the Society stated that to increase the resources and equipment of the Universities is one of the best ways of aiding research in pure Science. The Report of the University Grants Committee in February of this year indicated that the Universities were unable to meet their existing responsibilities, and that their resources are inadequate to meet legitimate demands upon them. It is, therefore, a matter of grave concern that the Government Grant to the Universities is now to be cut down heavily. The maintenance of the Universities at the level of efficiency which they have struggled so resolutely, and with much service but poorly paid, to sustain, will thus receive a very severe blow.

Regression is the more disappointing because, during the war, there came an awakening of the conscience of the nation in regard to Science. The national need for wider and deeper interest in, and understanding of Science came home to the community as it had not done hitherto. The importance to the nation of, for instance, the national Physical Laboratory, whose parent this Society may justly claim to be, began to receive more general recognition than before. Its importance to the State became cogent to the State. Six years ago saw the founding of the Advisory Council on Research to the Privy Council, and a year later the establishing of the Department of Scientific and Industrial Research. These were not created as part of the machinery for the war, though during that common need they, like every other national organization, made their contribution. They were brought into existence to remedy deep-seated shortcomings which the war revealed in the country's organization for scientific research. Their full effect was only to be expected to come now, after the attainment of peace. It is, therefore gravely disquieting that their State support estimates are being now reduced by some 30 per cent. and that further reduction still is asked for.

Again, if we turn to the domain of Biology, and take within that the field of Medical Science, the Medical Research Committee, as it then was, had been organised and started not long before the outbreak of the war. It had from its beginning shown its utility and brought evidence of the great field of usefulness before it. Its services during the war and since the ending of the war have been conspicuous, indeed inestimable. Public appreciation of it has enhanced. The Government has recently raised the status of the Committee, so that it is now the Medical Research Council under the Privy Council. Annual Reports indicate the quality and the volume of the work it is accomplishing. It is creating a new era of research in scientific medicine in this country. But its financial State aid is to be cut down for the coming year, and the extent of that reduction is a real anxiety to all who have at heart the progress of Medicine in this country and of the Sciences on which Medical Science itself rests.

I may say that, broadly taken, the apparatus for prosecution of research in this country is made up as follows: (1) Scientific and Professional Societies and some institutions entirely privately supported; (2) Universities and Colleges, with their scientific departments; (3) Institutions, using that term in the widest sense, directly subventioned by the State, such for instance as the Medical Research Council, the Development Commission, and the Department of Scientific and Industrial Research. Of these three categories, the first named, the Scientific Societies group, work without financial aid from the State, apart from the small though extremely useful two Government Grants distributed, mainly to individual workers, through this, the Royal Society. At the present time many of the Societies sorely need financial help to carry on their labours, and some are absolutely at a loss to know how to publish the scientific results that are brought to them. (2) The second category, the Universities and Colleges, depend in part upon Government aid. In the aggregate of twenty-one institutions of University rank, following Vice-Chancellor Adami's figures, students' fees and endowment provide about 63·5 per cent. of the total income; for the rest they are dependent on Government Grant. (3) The third category as said, draw State-support direct.

This triple system may seem a somewhat haphazard and inco-ordinate assembly. Yet in reality it is an organisation with much solidarity, and its co-ordination is becoming more assured. Its parts dovetail together. The first group, the scientific and professional Societies, is provided with a medium of intercommunication and co-action, the Conjoint Board of Scientific Societies. As to the separate categories composing the triple system itself, they also are in wide touch one with another. Between the Scientific and Professional Societies on the one hand and the Universities on the other, contact and inter-relation are secured by some degree of free and rightful overlap, both as regards general subject matter of research and of their *personnel.* Finally, there is excellent contact between both these categories and the third, the State-subventioned institutions. A special feature of the policy and administration of these State organisations secures this, a feature which makes the whole of this subject the more cognate to the purview of our own Society. To exemplify I may turn, for instance, to the Development Commission. Its programme of Fishery Research, avoiding the terms "pure" research and "applied" research, in view of the possible implication that pure research does not lead to practical result, directs research not alone to the solving of particular economic problems. It supports more especially what it terms "free" research, investigation in this case of the fundamental science of the sea and of marine life. This term "free" research is set in its full light by words of the Lord President of the Council, Mr. Balfour, where he points out that while the State may aid research, it will only destroy research if it resolves too rigidly to control it.

Again, with the Advisory Council of Scientific and Industrial Research, its programme, gradually defined during the past six years, is laid down as having four main points: (1) the encouragement of the individual research worker, particularly in pure science; (2) the organisation of national industries into co-operative research associations; (3) the direction and co-ordination of research for national purposes; (4) the aiding of suitable researches undertaken by scientific and professional Societies and organisations. It recruits researchers by giving financial opportunity to promising students to be trained in research attaching them to experienced researchers. In short, it apprentices to research a number of selected younger workers in Universities, Colleges, and other institutions scattered throughout the country.

So, similarly, the Medical Research Council. Its Secretary, Sir Walter Fletcher, in an illuminating presidential address to Section I of the British Association Meeting this summer, said, speaking of the nexus between scientific research and the progress of Medicine, "It is the accumulating knowledge of the basal laws of life and of the living organism to which alone we can look for the sure establishment either of the study of disease or of the applied sciences of Medicine."

It is evident, therefore, that with a policy based on such principles as these, the third category in the triple system constituting the organisation for scientific research in this country, is one which has common aim and solid touch with both the others, the Universities and the Scientific and Professional Societies. One sees in short that the organisation which has come into existence and is maintaining scientific research in this country, is a real organisation. It did not spring fully equipped from the head of Jupiter. It has grown up rather than been planned. In that respect it is an organisation essentially British, and it seems qualified to do its work for the country well. We hear of adventures, political and other, the offspring of the day. But these were no adventures, these, to my mind, welcome, long-overdue, steps forward by the State toward the succour of Science and its welfare, steps that help to strengthen and consolidate the organisation for research by such adjuncts as the Medical Research Council and the Department of Scientific and Industrial Research. One of the strengths of this organisation that has arisen is, in my view, that it interlocks with the educational system of the country. It is an organisation which proceeds on the wise premiss that, in the case of Science, the best way to get the fruit is to cultivate the tree. It is an organisation which is proving successful and economical. Its output has proved a more than liberal return on the funds at its disposal.

But essential to its own continuance is continuance of adequate financial support from the Government. A tripod cannot stand upon two legs. The State-contribution in this country is relatively not large, but it is most important. Important as it has been in the past, it has now an importance most especially great. The cost of investigation is now higher, much higher than it has been. Endowment funds carry less far than they did carry. Private benefactions and voluntary generosity, although willing, are less able to be found and less capable at this time; already gauged as inadequate of themselves alone before the War, they obviously cannot alone cope with the necessary undertakings now. The present is a time when a large-scale withdrawal of the Government's financial support must prove most formidably crippling. Such crippling will be greater than the actual measure of the sum withdrawn would entail in ordinary times.

None can fail to see the urgent need for national economy. It may be objected that the plea to which I am speaking is, in fact, one for the preferential treatment of Science. That is not so. Faced with need for stringent economy, there must, of course, be a rigorous cutting-down of expenditure that is unnecessary. But a first enquiry is the discrimination between expenditure upon the inessential and the essential. Otherwise the economies seemingly effected may be no economies. The savings may be made in a fashion most costly in the end. Conceded that there must be some reduction in the moderate State expenditure on research, it would be no true economy if that reduction were pushed to the point of causing collapse of the fabric for the production of much-needed knowledge or of whole compartments of that fabric.

The necessary supply of trained research workers cannot be retained or replenished except by a steady policy pursued. If the financial provision for research is too severely cut down, that will mean the extinction of various investigations which cannot be satisfactorily continued at all under narrower limits of expenditure than are imposed at present. One feature of modern research is that it has become more largely team-work, the combined effort of an assorted group of individuals with special training. Want of volume has tended to be a weak point in our national research. Reduction of the support by Government will react most rapidly on the number of competent investigators available, the number that makes a fair volume of team-work possible. The Report of the Advisory Council states that the effect of a set-back of this kind will be long-continued and adds that it may be lasting.

To pull down under emergency what has been built up through years of careful experience and is proving efficient, can hardly be ultimate economy. It is to unlearn a useful lesson learnt. Curtailment of the State aid—relatively small in this country—given to scientific research must harm the scientific production of the country. Some curtailment, however, at this time seems unavoidable. Though extension of buildings and equipment and *personnel* is wanted, it may be necessary to withhold that extension at this time, maintaining broadly the *status quo* ready for expansion when that is once more feasible. But if research be an indispensable factor in the rebuilding of the national life, sacrifices should not be required from it disproportionately greater than from other services of a similarly essential kind. Reduction of the State's support on a scale to entail ruin to the existent organisation would be a wastage rather than an economy. Calmly viewed, what more reminiscent of the wastage of the War itself than for machinery actually constructed, assembled, and producing what is needful for a nation's strength as a pillar in the industrial and intellectual temple of the world, to be now under temporary change abandoned or broken up; and at a time when industry as a whole stands convinced of scientific research as a necessity for its recovery and well-being.

My hope would be that scientific research on its present maintenance will be considered part of the intellectual bread of the community, part of the bed-rock on which rests the efficiency, not to speak of the industrial equipment of the nation; that it will be treated as such in the measure of State-support continued to it; that the State will remember that that support has to embrace at least both the Universities on the one hand, and, on the other, the research institutions administered by the State, for this reason, namely, that the country's organisation for research, complex in origin, yet economical and effective, stands as an integral system, to whose entire existence is essential an adequate State provision for both these constituent elements, indispensable, since they are, to the whole structure of the system.

I now proceed to the distribution of the Medals.

The Copley Medal is awarded to Sir Joseph Larmor.

Sir Joseph Larmor has long held a leading position in the British School of Mathematical Physics. There is hardly a branch of this subject to which he has not made contributions of distinct originality and great value. His earlier researches on Dynamics, on Optics, both geometrical and physical, and on Elasticity, are marked by keen insight and by the novelty introduced in the treatment of familiar subjects. In more recent periods he has written on problems of Geodynamics, with the same illuminating force. His contributions to the Theory of Electricity, in its many ramifications, are numerous and profound. His treatise on 'Æther and Matter' forms a distinct landmark in the history of the subject. In this we have the foundation of electromagnetic theory on the single principle of least action, with the electron taken into account as an æthereal structure. He was the first to establish (to the second order of velocity) the correspondence between moving and fixed electrical systems, and shares with Lorentz, the distinction of discovering the generality of this correspondence to any order. It may fairly be said that his preliminary work was of the utmost value in paving the way to the modern developments of the Theory of Relativity. In addition to his own researches Larmor has, as Lucasian Professor, stimulated the work of others with notable success. His intimate and extensive knowledge of the history as well as of the results of physical science marked him out as the appropriate editor of the works of Stokes, Kelvin, James Thomson, and Henry Cavendish, to which he has contributed most serviceable annotations.

A Royal Medal is awarded to Dr. Frederick Frost Blackman.

Dr. Blackman is distinguished for his contributions to plant physiology, and especially to knowledge of the process of photo-synthetic assimilation of carbon dioxide. In this connection he devised apparatus of great delicacy and accuracy. Later he proceeded to an exhaustive investigation on the rate of assimilation within the green leaf. He determined, under varied and controlled conditions, the inter-relationship of the external factors and their several and joint effects on the rate of assimilation, and has laid the foundation on which a good deal of subsequent work by other investigators has been rendered possible.

He was thus led to his theory of limiting factors, which has exerted much influence in both plant and animal physiology. With the help of his

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co-workers he has importantly extended our knowledge of permeability, and of the influence of anæsthetics on plants. He occupies a leading position amongst plant physiologists, not only by reason of the importance of his discoveries, but also on account of the effective stimulus he has given to the school of investigators who have been trained in his laboratory.

A Royal Medal is awarded to Sir Frank Watson Dyson.

Sir Frank Dyson is distinguished not only for his enlightened and energetic administration of the Royal Observatory, but by his many important contributions to Astronomy. He has devoted special attention to investigations of the movements and distances of the stars, and of the bearing of these upon the structure of the stellar universe. He has concentrated his energies particularly on the stars surrounding the north celestial pole, and has collected or determined for this region of the sky all the different data which seem likely to aid in the solution of the stellar problem. In a long series of papers he has shown himself able not only to conceive and execute large schemes of observation, but also to deduce by graphical and mathematical analysis the theoretical conclusions which are implicit in the mass of data. Some of his investigations are remarkable for the extensive data which have been utilised; one of them involves the proper motions of 12,000 stars, and another of 26,000 stars. These researches have given Sir Frank Dyson a place in the front rank of workers on stellar distribution and movements.

He has also given much attention to the accurate determination of stellar magnitudes, and has successfully established a regular programme of work on stellar parallaxes which has yielded results of high precision for a large number of stars.

Previous to this he had been conspicuously successful in obtaining records of the spectrum of the corona and chromosphere during eclipses of the sun; his publications on those subjects are among the most valuable sources of solar spectroscopic data. It was mainly to his foresight and organizing ability that we owe the successful observations of the deflection of light by the sun's gravitational field during the eclipse of 1919.

The Davy Medal is awarded to Prof. Philippe Auguste Guye in recognition of his work on optically active organic substances, on molecular association and on atomic weights.

In his early work on Organic Chemistry, Prof. Guye was led to investigate the question whether a quantitative relationship exists between the molecular rotations of optically active substances and their chemical constitution. Although the answer proved to be in the negative, the attempt to establish such a relationship was yet productive of much valuable research on optical isomerides in his own laboratory, and stimulated the efforts of many investigators in that branch of physical chemistry, particularly in this country.

Shortly after he had put forward his theory of the "product of asymmetry" he was attracted by the problems connected with Van der Waal's equation and the critical state, and, from his interest in these, two important lines of investigation opened out. The one had relation to the degree of molecular complexity of matter in the liquid state, and occupied his attention mainly between the years 1893 and 1911. The other led him at the beginning of the present century to advocate, with much energy and persistence, the advantages of the physical method of determining atomic weights. In this field of work he became one of the foremost investigators; his work on the calculation of precise gas densities was followed by chemical studies of the atomic weights of nitrogen, silver and chlorine, and by inquiries into sources of error, hitherto little recognised, in atomic weight determinations.

The Hughes Medal is awarded to Prof. Niels Bohr.

Prof. Bohr is well known to all physicists as the author of the conception to which the name "Bohr-atom" has been attached. A decade ago it became clear, from the researches of Sir E. Rutherford and others, that the atom of any element is formed out of an excessively minute positive nucleus of electricity, round which circulate a number of negative electrons equal to the atomic number of the element. Bohr discovered a mechanism for the motion of these electrons, which solved immediately the long-standing puzzle of the Balmer series of hydrogen, and which, after development and discussion, appears likely to provide a complete explanation of the spectra of the various elements. In this way he has opened up a line of investigation which has already attracted to itself many of the ablest mathematicians in Europe, and of which the success, in the simplest cases of the two light elements hydrogen and helium, is even now little short of perfect.

On the Optical Rotatory Power of Crystalline Ovalbumin and Serum Albumin.

By Elrid Gordon Young, Ramsay Memorial Fellow.

(Communicated by Prof. F. G. Hopkins, F.R.S.—Received July 6, 1921.)

(From the Biochemical Laboratory, Cambridge University.)

The need for a physical method by means of which it would be possible to recognise a chemical individual of the protein group of compounds has undoubtedly been one of the factors contributing to the difficulty of research into the chemistry of the proteins. The present methods of chemical analysis do not nearly approach sufficient accuracy to distinguish between successive recrystallisations of a protein substance. The first serious effort to prove that an individual protein could be isolated is to be found in the publication of Hopkins (1900). It was here shown that a protein could be prepared with a constant specific rotation for successive recrystallisations and for material obtained from different sources. Unfortunately, this desirable physical constant, which is independent of the degree of colloidal dispersion, has been shown to vary with variations in physical and chemical conditions. Thus Alexander (1896) found the specific rotation of certain globulins varied according to the concentration of the protein and of salt present. An investigation of this phenomenon by Pauli, Samec and Strausz (1914) confirmed the observations of Hopkins (1900) and of Osborne (1899) that the presence of neutral salts has no influence on the optical rotation of a protein. The addition of acids and of alkalies they found to increase the rotation of polarised light, while the degree of change depended upon the nature of the anion in the case of an acid and of the kation in the case of a base. These observations were made, unfortunately, with the mixture of proteins contained in ox or horse serum which had simply been dialysed until salt-free. The nature of these changes may find explanation in a tautomeric equilibrium of the lactam-lactim type in the protein main chain when in aqueous solution, as suggested by Robertson (1912) and Sörensen (1912), and to which view Pauli inclines.

$$\underset{\text{Lactam formula.}}{R{-}CO{-}NH{-}R} \rightleftarrows \underset{\text{Lactim formula.}}{R{-}C(OH) = N{-}R.}$$

The determination of the specific rotation of crystalline ovalbumin as a means of discovering its purity, as Hopkins had shown possible, was called into question by Willcock (1908), on the basis of the test used to show when the analysis sample had been washed free from sulphate. Using a ring test with

$BaCl_2$, Willcock claims that a very much greater sensitivity can be obtained; and by employing this method, specific rotations of different albumin crystallisations were found to vary considerably. If that fact were true, the biological chemist would still be without a means of recognising a chemical individual of the protein group, even if he had isolated one.

The experimental history as described in the following pages was developed with the object of discovering whether a constant specific rotation could be obtained for crystalline ovalbumin, and what was the relation between the hydrogen ion concentration and the optical rotation with reference to the isoelectric point. It was thought that such a study might yield an explanation of the variability of the rotatory power and possibly some direct evidence on the question of a tautomeric equilibrium in the protein molecule. These experiments are described first. The investigation was extended to a study of the conditions governing the preparation of a pure crystalline serum albumin and the properties of this substance examined.

EXPERIMENTAL.

The Specific Rotation of Crystalline Ovalbumin.

The values obtained for the specific rotation of hen ovalbumin by different observers at different times and with material under somewhat different physical conditions have been recorded in Table I. The earlier determinations on amorphous material are interesting in that they show a higher value than that observed for crystalline material. Osborne and Campbell (1900) have shown that the non-crystallisable albumin, which they have called conalbumin, has a higher value for $[\alpha]_D$ than the crystalline, and contamination by this substance probably explains the higher value.

Table I.

Observer.	Material.	Specific rotation.
Haas (1876)........................	Amorphous	−38°·08
Panormoff (1898)	,,	−36·2
Panormoff (1898)	Crystalline	−23·6
Worms (1898)......................	,,	−26·1
Bondzynski and Zoja (1894)...	,,	−26·0 to −42·54
Osborne (1899)	,,	−28·42
Osborne and Campbell (1900)	,,	−28·60 to −30·80
Hopkins (1900)	,,	−30·7
Willcock (1908)....................	,,	−30·3 to −31·6

Only the last three determinations are of importance, as they were made on similar material which had been recrystallised several times, and of these

Hopkins alone obtained a constant value for successive recrystallisations. The earlier figures were obtained from material crystallised but once, and by the inferior technique of Hofmeister in most cases.

The last worker on the subject claims that the true value is higher than −30·7° and attributes her average result of −31·0° as due to a more sensitive test for the presence of sulphate ions, thus allowing of more thorough washing of the coagulated albumin before drying and weighing. The technique which I have adopted in obtaining the following observations involved the use of a 400-mm. polarimeter tube, so that very large rotations were observed. A large Hilger polarimeter was employed and observations were made both with the sodium flame and with the green band of the quartz mercury-vapour lamp. The solutions used were made as concentrated as possible, being about 10 per cent. strength. The analysis of these solutions for their content of albumin was carried out in the first series by the Devoto method, using accurately calibrated pipettes for volume measurement of a quantity of solution sufficient to yield about 0·5 grm. of dried coagulum, and Kahlbaum's purest $(NH_4)_2SO_4$ in saturated solution as coagulating agent. This solution is naturally acid, having a P_H of 5·5 or less. Coagulation was brought about by placing the solution to which the albumin had been added for 1 hour on a boiling water bath. After cooling, the precipitate was filtered off on to hardened filter paper and washed with cold water until free from sulphate. Finally, the precipitate was removed quantitatively to a platinum basin by means of a few cubic centimetres of distilled water, the excess water was evaporated on a water bath, and the protein dried until of constant weight in a Lothar Meyer air bath at 110° C.

The removal of the last traces of sulphate from the precipitate is a difficult operation. I have tested the sensitivity of the $BaCl_2$ test for sulphates, applied as a ring and in the direct way, on a series of dilutions of normal H_2SO_4. When carried out as a ring test, using a saturated solution of $BaCl_2$ and allowing the tubes to stand 15 minutes before decision, the test is sensitive to one part in one million. The same limit was found for the test when applied by adding five drops to about 20 c.c. of filtrate and allowing to stand 15 minutes. An approximate idea of the quantity of sulphate being removed can be obtained in the final stage of washing by comparison of the turbidity obtained with that given by known strengths of H_2SO_4. By continuous washing, I have never been able to remove the last traces of sulphate in less than 4 days. This point is discussed below. If any protein goes back into solution during this long period of washing, the amount must be negligible, for I have repeatedly tested the filtrates for protein with negative results, and evaporated down quantities of the filtrate to dryness without obtaining any visible residue. Table II gives the results of a series of crystallisations:—

Table II.

Crystallisation.	Second.	Third.	Fourth.	Fifth.	Sixth.	Average.
Concentration (per cent.) ……	7·31	5·43	11·17	9·25	11·09	
P_H …………	5·3	4·9	4·9	5·1	4·9	
α_D …………	$-8·87°$	$-6·70°$	$-13·80°$	$-11·29°$	$-13·72°$	
α_E …………	$-10·80$	$-8·16$	$-16·73$	$-13·77$	$-16·73$	
$[\alpha]_D$ …………	$-30·34$	$-30·82$	$-30·87$	$-30·61$	$-30·89$	$-30·81°$
$[\alpha]_E$ …………	$-36·93$	$-37·36$	$-37·74$	$-37·34$	$-37·63$	$-37·53$

The values obtained for the specific rotation of successive recrystallisations is thus in very good agreement with that observed by Hopkins previously with similar material.

On account of the difficulty of washing free from sulphate encountered in the Devoto method, I have considered it of interest to compare this procedure with the simpler one of coagulating the sample for analysis in a buffer mixture at the isoelectric point. The most convenient buffer mixture for this purpose is a solution of acetic acid and sodium acetate in equal molecular proportions. Thus, 5 c.c. of a N/1 CH_3COOH solution were mixed with 5 c.c. of a N/1 CH_3COONa solution, and the whole diluted to 50 c.c. This solution has a P_H of 4·74. To this mixture was added the volume of albumin solution, containing about half a gramme of protein (5 to 10 c.c.). The containing vessel was then heated for half an hour on a boiling water bath, to coagulate the albumin completely. It was then allowed to cool, filtered off on to hardened filter paper, and washed until free from sulphate. The washing period is much briefer than when $(NH_4)_2SO_4$ is used as coagulating medium. But, even with this procedure, sulphate ions are very slowly removed after the first day's washing at the rate of about 1 mgrm. per 100 c.c. of filtrate.

Table III represents the specific rotatory power observed in a series of crystallisations by the use of both methods of coagulation simultaneously carried out, and it also shows a beautiful agreement between the determinations of the albumin concentration by the two methods used. The specific rotations obtained are particularly interesting, in that they are constant in magnitude after the second crystallisation, yet slightly lower than the series reported in Table II. This suggests two possibilities. There might be slight molecular differences in the albumin of two different lots of eggs hitherto undetected by our crude methods of analysis, or, what is much more probable, the crystals obtained in the first crystallisation might be a slightly different

Table III.

Crystallisation.	Second.	Third.	Fourth.	Fifth.	Average.
Concentration (per cent.)—					
(1) $(NH_4)_2SO_4$ method ...	9·87	10·95	15·43	12·64	
(2) Buffer method	9·89	10·96	15·42	12·66	
P_H	5·4	5·4	5·3	5·3	
α_D	−11·96°	−13·22°	−18·52°	−15·22°	
α_E	−14·66	−16·12	−22·58	−18·57	
$[\alpha]_D$	−30·26	−30·17	−30·02	−30·10	−30·14°
$[\alpha]_E$	−37·09	−36·79	−36·61	−36·70	−36·80

salt from that obtained previously. This possibility, more fully discussed in the notes on the method of preparation, suggested the desirability of studying the variation of the specific rotation in relation to the hydrogen ion concentration of the solution.

The Influence of P_H *on Rotatory Power.*

The condition in which the protein molecule is isoelectric to the electric current has such a profound influence upon its physical properties that it was thought that a study of the relation of this condition to the optical rotation might yield an explanation of the divergent values recorded in the literature. For the purpose of experimentation, crystalline material, which had been crystallised four to six times, was used. The last precipitate of crystals was centrifuged at high speed from its mother liquor and redissolved in distilled water. The resulting solution thus contained a small amount of $(NH_4)_2SO_4$. The changes in hydrogen ion concentration of the solution were followed by means of various indicators, using the standard buffer mixtures devised by Clark and Lubs (1917). A series of reagents was prepared by the use of material which had been purified to the standard required and dissolved in water which had been twice distilled in glass, firstly from $KMnO_4$ and H_2SO_4, and secondly from $Ba(OH)_2$. All test-tubes employed were of uniform diameter, of clear, colourless glass, and had been steeped in dichromate cleaning mixture for 24 hours, and then steamed in superheated steam for 1 hour.

After some experimentation, the following series of indicators was selected as most suitable for use in solutions containing both protein and salt:—

P_H range.	Indicator.
1·2— 2·8	Thymol blue.
4·4— 6·0	Methyl red.
6·4— 7·8	Neutral red.
7·8— 8·4	Phenol red.
8·4—10·0	Phenolphthalein.

To eliminate the possible errors due to the presence of proteins and salts, the method of dilution was adopted. Thus, 1 c.c. of fluid was pipetted into a test-tube, 4 c.c. of water (CO_2-free) added, and a suitable quantity of the indicator. A comparator was employed in the case of any turbidity or foreign coloration. This method of dilution was later verified by the use of the hydrogen electrode and potentiometer, and found to give correct values. The values obtained with the above indicators were also verified by the electrical method on test solutions. Methyl red, neutral red and phenol phthalein give very trustworthy figures.

Experiment 1.—A concentrated solution was quickly prepared from freshly crystallised material, filtered and placed in a 400-mm. tube. Its rotation was observed at intervals in a polarimeter for several days and found to remain absolutely constant from the first observation of −15·35°. The P_H of this solution was found to be 4·9. To this solution was added normal H_2SO_4 until the acidity was increased to P_H 3·75. Within a few minutes a very finely divided precipitate began to form which remained suspended and could be neither filtered nor centrifuged off. Further addition of acid so that the P_H became 2·7 merely increased the fine insoluble precipitate. A slow denaturation was apparently taking place and the solution was extremely sensitive to mechanical shock, immediately forming films of denatured material.

Experiment 2.—A fresh solution of P_H 4·9 was made alkaline by the addition of several drops of concentrated NaOH solution and the variation in the degree of rotation observed in a 200-mm. tube is recorded in Table IV:—

Table IV.

Date.	Temperature.	P_H.	α_E.
	° C.		°
December 10	11·0	4·9	−1·91
„ 13	11·0	4·9	−1·91
„ 15	11·0	4·9	−1·91
„ 15	11·0	6·65	−1·72
„ 16	11·5	6·65	−1·73
„ 19	12·0	8·4	−1·78
„ 20	12·0	8·4	−1·79
„ 21	12·0	8·4	−1·80

A distinct though small drop in rotatory power was observed and on further increasing the hydroxyl ion concentration the value for the rotation increased slowly.

The effect of adding concentrated NH_4OH to a fresh solution of albumin was next tried, employing a higher concentration of protein. Precisely the same phenomenon of a primary drop in rotatory power followed by a slow rise was observed as recorded in Table V:—

Table V.

Time interval.	Temperature.	P_H.	α_E.
	° C.		°
—	15·0	4·85	−4·25
24 hours	16·0	4·85	−4·26
10 minutes	15·0	9·15	−3·94
3 hours	15·0	9·15	−3·94
1 day	15·0	9·15	−3·95
2 days	15·0	9·15	−3·96
5 days	15·0	9·15	−3·99

Experiment 3.—The effect of both acid and alkali was demonstrated by means of the addition of very small quantities of HCl and NH_4OH and the rotations observed are recorded in Table VI:—

Table VI.

Time interval.	Temperature.	P_H.	α_D.	$[\alpha]_D$.
	° C.		°	°
—	11	5·3	−4·22	−31·89
5 minutes	11	7·5	−4·07	−30·76
30 minutes	11	7·5	−4·08	−30·83
2 hours	11	7·5	−4·08	−30·83
5 minutes	12	7·8	−4·10	−31·00
1 day	12	7·8	−4·22	−31·89
5 minutes	12	4·8	−4·40	−33·25
1 hour	12	4·8	−4·40	−33·25
2 days	13	4·8	−4·41	−33·33

The effect of the addition of alkali is most interesting and the prompt fall in the rotation followed by a slow rise to the original value is distinctly suggestive of a tautomeric equilibrium. I have observed this same phenomenon in the case of denatured serum albumin. The effect of increasing the hydrogen ion concentration of the solution is an instantaneous increase in the rotatory power, which remains constant at the new level.

The experiment was repeated in order to discover at what P_H the change in the rotatory power occurred, and to discover further if the change were reversible from either side of the isoelectric point. The observations are recorded in Table VII:—

Table VII.

Date.	P_H.	α_E.	$[\alpha]_E$.	Date.	P_H.	α_E.	$[\alpha]_E$.
		°	°			°	°
April 20 ...	4·9	−8·37	−37·79	April 20 ...	4·9	−8·37	−37·79
„ 20 ...	4·65	−8·53	−38·48	„ 20 ...	5·4	−8·09	−36·50
„ 21 ...	4·65	−8·58	−38·72	„ 21 ...	5·4	−8·10	−36·55
„ 22 ...	4·65	−8·63	−38·94	„ 22 ...	5·4	−8·11	−36·59
„ 23 ...	4·3	−8·74	−39·43	„ 23 ...	7·6	−8·14	−36·73
„ 26 ...	4·3	−8·72	−39·34	„ 23 ...	7·6	−8·15	−36·78
„ 30 ...	4·3	−8·73	−39·39	„ 25 ...	7·6	−8·14	−36·73
„ 30 ...	6·45	−8·06	−36·37	„ 26 ...	7·6	−8·20	−37·00
May 2 ...	6·45	−8·05	−36·32	„ 29 ...	7·6	−8·25	−37·23
„ 4 ...	7·1	−8·61	−38·85	„ 30 ...	7·6	−8·25	−37·23
„ 5 ...	7·1	−8·67	−39·12				

Note on the Crystallisation of Ovalbumin.

The *rôle* of acid in the process of crystallisation of albumin has received some attention at the hands of a number of investigators since the procedure was first carried out by Hopkins and Pinkus. These investigators found that a 10 per cent. solution of acetic acid served admirably for the adjustment of the solution so that crystals were deposited. Osborne recommended HCl very shortly afterwards, while Krieger found H_2SO_4 to give the best results. But as Hopkins pointed out in 1900 almost any moderately strong acid will bring about crystallisation. In his extensive study of the emulsoid colloid using crystalline ovalbumin as material, Sörensen (1917) employed N/5 H_2SO_4 with additional water and $(NH_4)_2SO_4$ solution.

In the course of my experience I have had to prepare many small lots of crystals and have employed several different acids of various strengths but always adjusting the reaction finally to about the same optimum hydrogen ion concentration. On several occasions I have found the method of Hopkins to fail to yield crystals at the beginning, although the two possible causes of failure which he mentions were excluded, viz., staleness of the eggs and insufficient whipping. If, after a heavy amorphous precipitate has been thrown down while standing after the usual adjustment, more acid be added, crystals sometimes appear on further standing. These failures I attribute to an increased alkalinity in the egg whites and inability to attain the proper hydrogen ion concentration for crystallisation before the protein was precipitated.

On two occasions when the optimum conditions had been established for crystallisation and a generous sowing of crystals added, a heavy precipitate was obtained, which consisted entirely of round globules of various sizes—the globuliths of the Hofmeister method of preparation. These globuliths could never be induced to crystallise, in spite of sowing and repeated agitation. They remained for two months in this condition, slowly coalescing into a hard gel formation at the bottom of the beaker. They were then redissolved and reprecipitated by $(NH_4)_2SO_4$, but again the precipitate consisted of nothing but globuliths of an enormous size, which again settled to a gel. This observation is of importance in the light of two recently published papers. Bradford (1920) claims to have crystallised gelatin, and Oswald (1915) albumin from human ascitic fluid, yet in both cases only globuliths were obtained. In Table VIII I have collected the amount of acetic acid used in crystallisation of several lots of egg white and expressed it in terms of cubic centimetres per 100 c.c. of filtrate:—

Table VIII.

Lot.	Fluid volume.	P_H.	Acid volume.	Product.
	c.c.		c.c.	
1	500	4·9	3·32	Crystals.
2	275	5·1	2·94	Crystals.
3	600	4·8	3·70	Globuliths.
4	650	5·0	8·56	Globuliths.
5	325	4·9	1·88	Crystals.
6	325	4·7	2·95	Crystals.

It is thus shown that the globulith mother liquors required more acid to adjust them to the conditions of precipitation than the normal. Now it has been observed by both Hopkins and Sörensen that this is necessary for eggs that are not strictly fresh. The phenomenon thus suggests to my mind further evidence toward an explanation of the *rôle* of acid and sulphate in crystallisation. We are dealing essentially with an equilibrium between albumin, water, salt and acid. The function of the salt is one of dehydration, as has been shown by Chick and Martin (1913). The function of the acid is probably twofold. By virtue of the fact that albumin is an ampholyte and on the alkaline side of its isoelectric point, it is bound to form a salt with free acid. At the same time, the acid ions have dehydrating powers. It is thus an adjustment of the available water molecules between the protein or protein salt, on the one hand, and the ammonium sulphate with the free acid ions, on the other. Sörensen (1917) has brought forward evidence from careful quantitative experiments to show that crystalline ovalbumin is a definite hydrate. The above-mentioned observations would be then comprehensible if, due to slight autolytic changes, the protein had lost some NH_2 groupings, and thus at least some of its power of salt formation with acids, yet retained its ability to form a definite hydrate. The globuliths, on this view, would be egg-hydrate, but not potentially crystalline ovalbumin.

In the light of the above discussion it is of interest to consider the conditions under which Hofmeister first obtained crystals. He slowly concentrated a half-saturated solution of ammonium sulphate containing albumin. Now, such a solution is naturally acid, due to the fact that $(NH_4)_2SO_4$ is a salt formed by the union of a very strong acid with only a moderately strong base. Furthermore, on slow evaporation in open vessels some of this free NH_4OH will be lost, and the solution will become more acid until such a point is reached at which the hydrolysis will become negligible. It would thus be conceivable that at one stage of evaporation globuliths would appear, and, at a later stage, when the solution had become more acid, crystals. The globuliths might then take up more acid and crystallise, since the phases differ in degree of their constituents and not in kind, and the disperse phase is more or less permeable to electrolytes in the continuous one.

In any case, both crystals and globuliths are precipitated again from a solution of $(NH_4)_2SO_4$, which is much less concentrated than that required for ordinary amorphous material. The concentration of $(NH_4)_2SO_4$ is usually slightly more than quarter saturation, while amorphous albumin in equal concentration requires considerably more than half saturation. This fact in itself, to my mind, is a very good reason for believing that both crystals and globuliths are hydrates of ovalbumin. The *rôle* of $(NH_4)_2SO_4$ would thus be to remove the solvate water associated with the albumin molecular aggregates which kept them in solution, but to leave the hydrate water still attached. If the hydrate were not formed, then the concentration would have to be considerably increased in order to control this excess of free-water molecules, and an amorphous precipitate would result.

Discussion of Results.

From the experiments described in the preceding sections we have seen that there is a variation of the specific rotation of ovalbumin, depending upon the hydrogen ion concentration of the solution. The addition of acid to a solution at its isoelectric point causes an increase of its rotatory power, which remains constant. The addition of alkali to a similar solution causes a prompt fall in rotatory power, which slowly rises to the original value. Further addition of alkali has no effect. These changes are reversible from either side of the isoelectric point. If, however, the albumin sample be kept at the isoelectric point or thereabouts, without adding either acid or alkali, it is possible to obtain a constant specific rotation for successive recrystallisations.

The maximum experimental errors are small. The polarimeter readings are accurate to 0·01 of a degree, and since rotations of ten degrees or more have been observed, the error from this determination would not be greater than 0·02 per cent. The error in the estimation of the dried coagulum from variations observed might amount to 2·5 mgrm. in 0·5 grm., or a maximum error of 0·5 per cent. A fluctuation of 0·25 of a degree in the specific rotation value would be the maximum experimental error permissible. It will be found that the results agree more closely than this as a rule. The variations in specific rotation produced by variations of P_H, as shown in Table VII, for example, amount to several degrees. There seem to be two possible explanations for this phenomenon. As Fischer has shown, the salts of amino acids have different optical rotations from the free acids themselves. Now the isoelectric point must be taken as the natural point of neutrality of the protein molecule; that is, the point at which the dissociation constants of the protein as an acid and as a base balance. By the addition of acid or alkali, salts would be formed on amino acid groupings, and the measure of salt formation would be the governing factor in change of optical rotation. This is certainly not so on the alkaline side, for beyond the first drop in rotation, further addition of alkali has no obvious effect. Likewise the addition of acid can change the rotation once, and once only, apparently.

The second explanation, as suggested by Robertson, that a tautomeric equilibrium exists in certain amide groupings is most forcibly emphasised by the slow change in optical rotation in alkaline solution, and, further, by the reversibility of the phenomenon generally from either side of the isoelectric point. That such a tautomerism is probable from other reasons has been very fully shown by Robertson, in supporting his theory of protein ionisation. Furthermore, such an equilibrium would be governed by the hydrogen ion concentration, and not by the quantity of acid molecules present. The slight changes in the acidity of the medium might thus account for the fluctuations of the specific rotations observed and recorded in the literature.

Crystalline Serum Albumin.

Crystalline serum albumin was first prepared by Gürber in 1894 by the direct application of Hofmeister's method to horse serum. Merely from the appearance of the crystals, Gürber concluded that three albumins occur in serum, all crystallisable. Michel, in 1896, introduced a slight modification in the technique, and determined the coagulation temperature and specific rotation of the crystalline product. Hopkins and Pinkus (1898) mention the fact that it is possible to obtain crystals from serum by the acid method, but the time required is one to several days. Krieger (1899) shortly afterwards suggested the use of H_2SO_4 in place of acetic acid without any quantitative data. In his paper on pure ovalbumin, Hopkins mentions that, in some attempts to purify serum albumin in like fashion, more difficulty was experienced in arriving at a homogeneous product by reason of pigment contamination. The last study of serum albumin was carried out by Hardy and Gardiner, and only published in abstract form. By means of absolute alcohol and anhydrous ether as extraction solvents and dehydrating agents at −4° C., a snow-white protein was obtained, which could be readily dissolved and crystallised. This mode of preparation serves to remove all traces of fatty material present in the original precipitate from serum, and has been used in part of the experimental work. The claim is made by these authors, unfortunately on very little published experimental evidence, that the albumin in serum exists as a complex of albumin, cholesterol esters and two pigments. This point is discussed later in the light of my experimental findings.

The various values recorded in the literature for the specific rotation of serum albumin are given in Table IX.

Table IX.

Observer.	Material.	Specific rotation.
		°
Frédéricq (1880)	Amorphous	−56·07 to −58·41
Starke (1881)	,,	−60·05
Sebelien (1885)	,,	−60·1 to −62·6
Michel (1896)	Crystalline	−61·0
Maximovitsch (1901)	,,	−47·47

Preparation of Material.

Two methods of preparing albumin have been followed with a view to contrasting the products obtained, the alcohol-ether method of Hardy and Gardiner, and a procedure evolved from the general principles of the Hopkins-Pinkus method. The blood was drawn from the right jugular vein of a normal horse and collected in sterile winchesters, where it was allowed to clot spontaneously. The serum was siphoned off after 2 or 3 days. With one preparation the blood was first defibrinated, and then centrifuged free from corpuscles.

Method 1.—The serum obtained was transported to cold storage chambers, where it was poured into three times its volume of 95 per cent. alcohol at a temperature of −4° C. The proteins thus precipitated were allowed to stand for 24 hours, when filtration was commenced through folded filters. The bulky cream coloured precipitate was washed at −4° C. as follows: (1) three times with 95 per cent. alcohol; (2) twice with absolute alcohol;

(3) twice with absolute ether. The precipitate was next transferred to soxhlet thimbles, placed in a desiccator, and transported to the soxhlet apparatus. In this it was now extracted with absolute ether for 15 hours, after which there was no further loss in weight. The thimbles were quickly removed to a desiccator containing H_2SO_4 and thoroughly dried *in vacuo.* On cooling, cholesterol esters crystallise out from the ether used in extraction. The latter apparently extracts some pigment as well, for it becomes coloured a bright yellow. The product obtained by this procedure is a very fine snow-white powder, which can be kept indefinitely if thoroughly dried. It is necessary to keep the precipitate well protected from moist air when in contact with either alcohol or ether at a temperature above 0° C. The precipitate will otherwise turn into a brown, brittle mass on drying, which is insoluble in water. From 3250 c.c. of serum, 240 grm. of protein were obtained, representing approximately 7·4 per cent:—

Analysis of Material.

The white powder in dry form, or as a concentrated solution from which the protein had been removed by means of dialysed iron, was examined for the following substances by the tests mentioned below with negative results in every case.

(1) Cholesterol by Salkowski and Libermann-Burchard.
(2) Lipoid by organic phosphorus, choline and sulphuric acid.
(3) Fat by acrolein.
(4) Protein hydrolytic products by biuret.
(5) Glucose by Benedict's picric acid.

The powder was analysed for total non-volatile solids and for its ash-content by heating to constant weight in a platinum crucible and igniting at a low temperature. The average for three determinations of loss in weight, probably representing volatilisation of traces of alcohol and ether, was 12·36 per cent. and of ash 1·34 per cent. on the dry weight. The samples of powder remained soluble in water in spite of having been subjected to a temperature of 112° for 8 hours. This was no doubt due to the thorough dehydration.

By reason of the statement made by Hardy and Gardiner that the product obtained by them from horse serum after a somewhat similar procedure showed the normal alkalinity of serum, which, they remark, is not due therefore simply to alkaline carbonates, it was deemed important to determine the alkalinity of the powder in solution. A solution of 13·9 per cent. strength was placed in a hydrogen electrode vessel of the Barendrecht type, installed in a constant temperature chamber at 18°, connected up with a calomel electrode containing saturated KCl solution and the usual electrical appliances for the electrometric determination of hydrogen ion concentration. The Walpole medical research potentiometer was used with a Weston standard cell as standard. The E.M.F. observed was 689·5 millivolts which represents a hydrogen ion concentration of $2{\cdot}45 \times 10^{-8}$ or P_H 7·61. The solution was next diluted with an equal volume of water and the difference of potential again determined. The result was the same as before.

As the ash-content of the protein powder is quite appreciable, and as is evident from the experiments recorded below this ash is made up, at least in part, of fixed alkali, the alkalinity of the solution I would attribute to the inorganic constituents rather than to the weakly basic protein.

In the following experiments on the albumin obtained by the above method the determinations of P_H were made by the potentiometric method as used for the alkalinity determination.

Experiment 1.—A 20 per cent. solution was prepared in distilled water. It may be noted here that in spite of its content of globulin the protein powder is entirely soluble in distilled water. It would thus appear that by this method of preparation the eu-globulin is so changed that it is now quite soluble in distilled water. To the mixed protein solution was added a saturated solution of Kahlbaum's purest $(NH_4)_2SO_4$ in equal volume. As is the case with ovalbumin preparations a distinct odour of ammonia is perceptible, indicating the presence of fixed alkali. The P_H of the medium was 5·84. The globulin precipitate was allowed to settle for 24 hours. It was then filtered off and a portion of the filtrate made acid with acetic acid (10 per cent.) to the point of equilibrium found desirable in the crystallisation of ovalbumin, *i.e.* a slight permanent precipitate. No crystals were formed and the solution had a P_H of 4·16. Another portion of the filtrate was similarly treated with acid but to a less degree. Crystals appeared in 12 hours. The P_H of the mother liquor was 4·30. The remaining filtrate was treated with acid to a point when the first faint turbidity was visible. Crystals in abundance were deposited inside of an hour. The P_H of the mother liquor was 4·54. The angles of the crystals were sharp and the crystals themselves large and single, resembling small hippuric acid crystals very closely. They were filtered off and dissolved in distilled water. There was absolutely no trace of residue remaining in the fluid. The solution was coloured a yellowish-brown. An attempt to recrystallise this preparation by addition of saturated $(NH_4)_2SO_4$ solution, added to the point of the first permanent turbidity, produced not a single crystal but only a bulky amorphous precipitate in about 30 minutes.

Experiment 2.—A 15 per cent. solution was prepared and the globulin removed as previously. Acetic acid was added until the P_H was adjusted to 4·8. Crystals began to form in 2 minutes and by 5 minutes there was a heavy precipitate, consisting entirely of crystalline material. The amount of albumin which was obtained in crystalline form by this procedure was estimated at 70·4 per cent. The crystals were redissolved in water and $(NH_4)_2SO_4$ solution added with great care at a very slow rate to a point somewhat short of a permanent precipitate. On standing, the solution deposited crystals in about an hour, and the yield was increased by slow addition of $(NH_4)_2SO_4$. The crystals were again brought into solution and the liquid still showed a slight yellow colour. The rotatory power was observed and the concentration of albumin determined by the Devoto method, as previously described. The albumin was again recrystallised and the determinations repeated. Table X summarises the results, and it is thus evident that the specific rotation is constant after the first crystallisation:—

Table X.

Crystallisation.	Protein concentration.	α_D.	$[\alpha]_D.^{13}$	α_E.	$[\alpha]_E.^{18}$
	per cent.	°	°	°	°
—	0·98	−1·13	−58·06	−1·36	−69·26
First	2·18	−2·74	−62·94	−3·41	−78·20
Second	3·36	−4·29	−62·84	−5·26	−78·32
Third	1·42	−1·78	−62·70	−2·22	−78·25
Average	—	—	−62·83	—	−78·26

Method 2.—The object of this experiment was to discover whether it was possible to obtain an albumin giving the same specific rotation, but prepared by a different method. The second method of obtaining serum albumin in a pure state was evolved from attempts to obtain crystals from serum by the exact application of the method of Hopkins for ovalbumin. This method has been used by several investigators as a means of obtaining relatively pure serum albumin for various purposes, but the procedure has never been carefully studied, and it was still an open question whether an individual albumin could be obtained from serum. Furthermore, the fundamentally important relations of serum albumin to the lipoids, cholesterol, and cholesterol esters of the blood stream seemed possible of investigation by a contrast of the serum albumin obtained directly and by the isolation method previously described.

The method was constructed on the basis of a series of preliminary experiments with both CH_3COOH and H_2SO_4 in an endeavour to determine the optimum P_H conditions. It soon became evident that any amorphous precipitate which was formed before crystallisation never became crystalline. This is in sharp contrast with the behaviour of ovalbumin. The conditions for the deposition are spread over a much wider range of concentration both of hydrogen ions and of ammonium sulphate. Crystals, moreover, will continue to form as very long narrow needles for several weeks after their first appearance.

When the first crop of crystals has been obtained, centrifuged off and placed in distilled water, there is always a considerable residue, although no amorphous material can be seen amongst the crystals. The nature of this residue has been investigated and is discussed in a later section. In order to discover whether the insoluble residue remaining after the first crystallisation could be eliminated by previous washing of the serum with ether, this experiment was tried on a sample of serum with complete success. The crystals formed with greater ease, probably due to the lowering of the surface tension at the interfaces of growing crystal and mother liquor. The angles of the crystals were very sharp and the size was uniform; furthermore, the crystals dissolved rapidly in distilled water, leaving only a minute trace of insoluble matter.

The procedure which I have found successful for obtaining a large uniform yield of crystals and for recrystallisation is as follows.

The serum is washed with an equal volume of ether in several small quantities by the use of a separating funnel. Serum globulin is removed by addition of an equal volume of saturated $(NH_4)_2SO_4$ solution. The precipitate is filtered off after 4 or 5 hours. The orange-yellow filtrate is slowly acidified by means of acetic acid (10 per cent) or sulphuric acid, N/3, to the point of the first trace of turbidity, the solution being constantly stirred. This point corresponds to a P_H value of 6·0, and a pigment change from red to yellow somewhat precedes it. The cloudiness will gradually become more dense as further masses of crystals separate. After two hours the acidity is further increased by the addition of about half as much acid as previously added. Crystals will be found to form with much greater ease once their deposition has been induced at a low hydrion concentration. After another two hours a third portion of acid is added, equal to the second. This should establish the P_H of 4·9 to 5·1, below which it is not advisable to go. The yield of crystalline material may be further increased by addition of more $(NH_4)_2SO_4$ solution, a few cubic centimetres at a time (1 c.c. per 100 c.c. of fluid) until precipitation ceases. The crystals so separated go into solution practically without residue, but the solution is strongly pigmented. Crystallisation can be repeated without much loss of material if care is used in adding the $(NH_4)_2SO_4$ with sufficient slowness. This operation is more difficult than the corresponding one with ovalbumin in that to produce a permanent precipitate would be quite fatal to crystal formation. $(NH_4)_2SO_4$ as a saturated solution must be added to a point when the precipitate, which first forms temporarily, redissolves slowly. From this on it is safest to add only a few cubic centimetres every 20 or 30 minutes (1 c.c. per 100 c.c. fluid). By this method I have obtained 82 per cent. of the total albumin in crystalline form. I have crystallised serum which had remained for six months at $-4°$ C., so that the factor of staleness would not appear to affect the crystallisation of serum albumin to the extent that it does that of ovalbumin.

Again, I would emphasise that, as in the case of ovalbumin, we are obviously treating an equilibrium reaction which is difficult to force to completion. The albumin remaining in solution need not of necessity be looked upon as non-crystallisable or as essentially different from the crystalline. It may be that, if it were possible to control physically all the constituents of the reaction, and to alter these as they were changed by the deposition of crystals, then the albumin obtained in crystalline form would amount to nearly 100 per cent. The physical difficulties, however, become greater as the concentration of albumin in solution becomes less. The fraction of total albumin crystallised, viz., 82 per cent., is somewhat higher by this method than that obtained by the first method, viz., 70 per cent. In the first method, however, the concentration of albumin was much lower, owing to the small amount of material available. It was thus more difficult to control the distribution of water between remaining albumin, $(NH_4)_2SO_4$, and free acid, while maintaining the latter at its optimum concentration.

By the above method, it has been possible to carry through a series of crystallisations with determinations of rotatory power. Table XI summarises the results obtained :—

Table XI.

Crystallisation.	Temperature.	Concentration.	$[\alpha]_D$.	α_E.	$[\alpha]_E$.
	° C.	per cent.	°	°	°
First	20	5·00	—	−3·73	−74·78
Second	17	5·41	—	−4·09	−75·71
Third	15	6·08	−62·98	−4·77	−78·60
Fourth	20	2·34	−62·70	−3·08	−78·24
Average	—	—	−62·84	—	−78·42

The third and fourth values are practically identical when the limits of accuracy of the determination of the albumin are considered. It is, moreover, of great interest to note that the value coincides with that obtained by the alcohol-ether method, as recorded in Table X.

Discussion of Results.

There has been much discussion for years past as to the possible chemical alliance of proteins with fats or lipoids in the blood. This is particularly true of eu-globulin, and Hardy and Gardiner have briefly indicated that it is possible that serum albumin is chemically bound to cholesterol esters and pigments in serum. Now, it is necessary to recognise that, in determining the value for specific rotation, we really have only allied rotatory power with coagulable material. That is to say, that identical weights of coagulated material in undenatured form from the two preparations, if made into solutions of identical concentration, would have an identical rotation. If cholesterol were present in chemical union with one albumin, then it must of necessity have an inappreciable effect upon the total observed rotation, and be completely volatilised during the drying operation. By reason of the fact that cholesterol is optically lævo-rotatory, these circumstances are, to say the least, improbable. One other possibility must be mentioned. The cholesterol, or other fatty material with which the albumin was chemically associated in serum, might be split off in the act of crystallisation. The "milieu" for such a chemically conceived separation is, indeed, nothing more vigorous than a concentrated $(NH_4)_2SO_4$ solution and very dilute acetic acid at a temperature of 20°. At the same time, proteins are extremely labile molecular structures. There is, furthermore, one piece of concrete evidence. On first crystallisation direct from serum, there is an insoluble residue of a fatty nature. The deposition of this residue is completely inhibited by previous ether extraction. The crystals themselves are most probably a pure protein, and of the same nature as found for ovalbumin, although differing in their chemical structure of amino acids, as indicated by the difference in specific rotation.

The solutions used for the determinations of specific rotation recorded were never entirely free from pigment. The solutions from the first method were much clearer than those from the second. In the latter, the successive recrystallisations diminished the pigmentation of the solution but little. I have tried many methods of removing pigment by adsorbents without success, *e.g.*, kieselguhr, charcoal and freshly precipitated barium sulphate. If the crystals are produced very slowly, the first crop formed is always much more coloured than subsequent ones. The amount of pigment, however, must be minute, since concentrations of over 5 per cent. of albumin can be examined in 200 mm. polarimeter tubes with ease. The work of Palmer and Eckles (1914) tends to show that the pigments are carotin and xanthophyll of plant tissues. But since carotin, which predominates greatly in horse serum, has such an intense colouring power, the amount in the solution used optically must be very small—less than 1 mgrm. in 100 c.c.

The true relationship between the fatty material or pigment and the protein in natural serum is best understood, to my mind, by the concept that in such complex compounds as the proteins there is no sharp distinction between the so-called physical adsorption and chemical union of associated substances. As Langmuir has pointed out from inorganic studies adsorption is fundamentally chemical.

The Insoluble Residue.

I have examined the residues which remained after the first crystallisation of several lots of albumin from serum. It is very obvious that they differ markedly in texture. Occasionally they can be collected with difficulty on account of being an oil. As such it is impossible to centrifuge and difficult to filter. When separated it is found to be entirely and readily soluble in ether, except for a little contaminating protein. This solution shows a very heavy precipitate on adding acetone but an oil is deposited on slow concentration. At other times the residue is a solid which can be readily centrifuged off and washed free from pigment. I have dissolved this in ether and hot alcohol. On cooling the solution in alcohol deposits, needles of the appearance of cholesterol esters and the solutions give a very strong cholesterol reaction.

Since no amorphous material is ever visible under the highest power of the compound microscope when the first crystalline product is examined, yet a very marked residue remains on attempting resolution, I examined the crystals while they were going into solution under the microscope. It then became clear why the albumin dissolved so slowly. As the highly refractive protein interior of the crystal disappeared there still remained the outline of the crystal. Soon the field became covered with these shells of the former crystals without any remaining protein. There was still no amorphous material. On agitating the slide, however, the crystal outlines rounded into fat-like globules. It would thus appear that each crystal had had a thin covering of fatty material deposited about it. This would naturally interfere with the process of crystallisation and make the crystal sizes vary. It is noticeable that large crystals are first formed which are four or five times the mass of smaller ones appearing later. Furthermore the presence of this fat-like material alters the shape of the crystal, obliterating angles and inhibiting rosette formation. When the serum is ether-washed previously to crystallisation, the crystals show sharp angles, are of uniform size and more quickly deposited.

The above observation shows how the nature of the medium may affect the structure of the crystals and is of interest in the consideration of all the work which has been done in the endeavour to link up crystalline form of proteins with plant or animal species from material which has been crystallised but once from its natural medium. It is also interesting in showing the possible physical associations between lipoid and protein material in protoplasm and the difficulty of removing the one constituent without affecting others.

SUMMARY AND CONCLUSIONS.

Crystalline Ovalbumin.

The specific rotation of crystalline hen ovalbumin is found to be $-30{\cdot}81^\circ$ for $[\alpha]_D^{15}$ and $-37{\cdot}53^\circ$ for $[\alpha]_E^{15}$. The value for $[\alpha]$ is constant within the limits of experimental error after the second crystallisation if recrystallisation be carried out about the isoelectric point (P_H 4·9–5·1).

At a lower hydrion concentration (P_H 5·3–5·4) a constant lower specific rotation is obtained, $[\alpha]_D^{15} = -30{\cdot}14^\circ$ and $[\alpha]_E^{15} = -36{\cdot}80^\circ$. The results were identical by two different methods of analysis.

The optical rotation of an albumin solution at its isoelectric point remains constant. If it be made slightly acid a prompt rise in rotatory power is observed to a new constant level. If the solution be made alkaline to P_H 4·9 a prompt fall in rotatory power ensues which very slowly rises to the previous value. This phenomenon can be brought about from either side of the isoelectric point and is reversible. The variations in optical rotation are explained on the basis of a tautomeric equilibrium of the lactam-lactim type.

Experiments are recorded which tend to show that globulith formation is no indication of the power of a protein to crystallise, but probably indicates hydrate formation.

Crystalline Serum Albumin.

Two methods have been used for the preparation of pure serum albumin (horse). The first method involves the precipitation and complete dehydration of the mixed proteins of serum at a temperature of -4° C., by means of absolute alcohol and ether, removal of fatty substances by Soxhlet extraction, and crystallisation of albumin from aqueous ammonium sulphate solution; 70·4 per cent. of the total albumin was obtained in crystalline form. After the first crystallisation the product possesses a constant rotatory power, $[\alpha]_D^{18} = -62{\cdot}8^\circ$, $[\alpha]_E^{18} = -78{\cdot}3^\circ$.

The second method involves the extraction of the serum by ether and the crystallisation of the albumin by ammonium sulphate and acid at P_H 6·0 to 5·0; 82 per cent. of the total albumin is thus obtained crystalline. After the third crystallisation the specific rotation is constant, $[\alpha]_D{}^{18} = -62{\cdot}8^\circ$, $[\alpha]_E{}^{18} = -78{\cdot}4^\circ$.

The insoluble residue from the first crystallisation from natural serum is shown to be a deposit of fatty material around the crystal surface. Its nature varies with different preparations, sometimes phosphatides predominate: sometimes cholesterol esters. The relation of fatty material and protein in serum is discussed.

In conclusion I wish to express my sincere thanks to Prof. F. Gowland Hopkins for his valuable advise and criticism during the course of this research.

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Experiments on Amphibian Metamorphosis and Pigment Responses in Relation to Internal Secretions.

By JULIAN S. HUXLEY (New College, Oxford) and LANCELOT T. HOGBEN (Imperial College of Science, London).

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The problem of metamorphosis in Amphibia attracts attention from many points of view. These organisms have, one may say, acquired interest as being indicators for the action of certain internal secretions; the underlying mechanism of metamorphosis is still imperfectly understood; and the general biological problems involved, especially with respect to the neotenous and perennibranchiate forms, are remarkably fascinating. The experiments here recorded were undertaken with a view to elucidating these issues more fully, and in the course of them data relevant to the pigmental responses of Amphibian larvæ also emerged. For various reasons it was deemed desirable to publish an account of the observations so far completed. Acknowledgment is made to Mr. D. F. Leney of New College for assisting in the care of animals, to the Royal Society and the Trustees of the Dixon Fund for grants in aid of the expenses incurred by the authors respectively, and to Prof. E. W. MacBride, F.R.S., for his kindness in reading the MS.

1. *Metamorphosis of the Axolotl by Thyroid Administration.*

(*a*) When a note (Huxley, 'Nature,' 1920) on the metamorphosis of medium-sized but immature Axolotls by means of thyroid feeding was published by one of the authors a year ago, and confirmed a few months later (Hogben, 'P.Z.S.,' 1920), our attention had not been called to Jensen's work (1916). Since this appears to be little known and is not readily accessible to English-speaking workers, it is proposed to give a short *résumé* of some of his most important findings.

First, however, a record of one of our own experiments will be given. Two black Axolotls, one male, the other female, and both sexually mature (though not quite full-grown), were kept in a large tank, together with two fine full-grown white Axolotls as controls. The controls were fed on worms, while the others were given pieces of ox-thyroid. In every case the feeding was controlled, the worm or piece of thyroid being kept near the animal's mouth until swallowed; if persistently refused, it was removed. The thyroid feeding began on November 30, 1919. On December 29, the thyroid-fed animals were past the critical stage of metamorphosis (see Boulenger, 1913); on January 13 no trace of gills was left; on January 19 the animals left the water, and the metamorphosis could, therefore, be considered as complete. The temperature was at first that of the room, varying from 8° to 15° C.; from January 2, when the water was warmed by an electric-light bulb, it rose to between 14° and 16° C. The length of the specimens at completion of metamorphosis was 17·4 cm. (♂) and 14·6 cm. (♀); the male had the typical sign of maturity—enlargement of the lips of the cloaca—well-marked. The controls during the same period showed no changes. It is an interesting fact that the metamorphosed animals, although tending to come more frequently to the surface about the time that the critical stage was reached, showed no inclination to leave the water until after the morphological alterations had run their course. It is only when a few days have elapsed after the completion of the visible external changes that the animals emerge from the water.

(b) *Jensen's Work.*

Curiously enough, Jensen also believed that he was the first to obtain the metamorphosis of Axolotls by thyroid, and it was only after his paper was completed that he found that he too had been anticipated—by Laufberger (1913). Jensen's experiments, however, are much fuller. He used calf-thyroid as diet. Two-week old larvæ on this diet all died within a few days, without change of form. Four immature specimens (10 to 16 cm. in length) metamorphosed in 17 to 25 days without exception. They were fed only three to eight times, with 1 to 3 grm. thyroid in all. An intra-abdominal injection of iodothyrin caused metamorphosis in the same length of time (10 specimens). The amount of iodothyrin injected had no influence upon the rate of metamorphosis within the limits used (1 to 10·5 mgrm.). One specimen *fed* upon iodothyrin started to metamorphose, but after 21 days of very slow change began to reverse the process, and became larval again. Four mature specimens, 22 to 24 cm. long, and two of them known to be over 6 years old, were also successfully metamorphosed. The time required was here greater, varying from 27 to 32 days. Two of these animals were females who had just started to lay eggs; the very interesting fact was noticed that oviposition ceased almost immediately the thyroid diet was started. (It would thus appear that the katabolic action of thyroid will not permit of the anabolic activities necessary for the production of eggs. On the other hand, the experiment does not prove that egg laying *cannot* proceed during metamorphosis, for the amount of thyroid given was certainly very much in excess of that necessary to produce metamorphosis. It would be of great interest to attempt work along these lines with amounts of thyroid (or iodothyrin or thyroxin) close to the critical amount.) One immature animal was fed on thyroid while in deep water; in spite of this, metamorphosis proceeded at the usual rate. There were no failures to metamorphose (except the above-mentioned one on iodothyrin *diet*) among all the survivors, and the two that died under the treatment were metamorphosing regularly up to the time of their death. *Salamandra maculosa* larvæ were also metamorphosed by a thyroid diet. Eight-day larvæ came out on land in 13 to 16 days, and the same period of time was taken by 30-day larvæ. Axolotls in 1/10,000 KI solution did not metamorphose, but on the contrary developed abnormally large gills.

Experiments were also carried out by Jensen upon Necturus and Proteus. One adult Necturus was at first fed on thyroid, later injected with iodothyrin. The only changes noted were the resorption of one gill on one side, and a slight change in pigmentation. The animal died after some time. Four specimens of Proteus were also treated, some fed on thyroid, others injected with iodothyrin. The animals were 12 to 24 cm. long. In all cases, there was a distinct but slight atrophy of the tail-fin and of the gills, but the total shrinkage of the fin in height was not more than 1 to 1·5 mm., and after 6 months it regained its original size. Thus, with animals of this size, only slight effects could be obtained, indicating that in these permanently neotenic forms, the tissues are not sensitive to thyroid in the same way as in normally metamorphosing or facultatively neotenic species. Similar treatment failed to produce metamorphic effects in the Ammocœte larva of the Lamprey.

Jensen further notes that in many, but not all, of his treated Axolotls, pathological symptoms occurred as a result of thyroid or iodothyrin. The animals refuse to eat, are usually sluggish when stimulated, rush about madly for several minutes, and exophthalmos of a very pronounced type appears. This exophthalmos, it is interesting to note, disappears with the other pathological symptoms from a few days before metamorphosis to three weeks after it has occurred.

(c) *External Agencies, Age, and Rate of Metamorphosis.*

As will be seen later, our young Axolotl larvæ fed on thyroid, all metamorphosed in from 24 to 32 days. We are thus in a position to compare the time necessary for metamorphosis induced by thyroid-feeding with that induced by air-breathing. Marie von Chauvin's classical experiments were done with great precautions, and the time taken was very large. Those of Boulenger (1913) will serve as our example, for it is not probable that the time for metamorphosis by this means can be much reduced beyond what was found by him. His results show that the time required for metamorphosis by air-breathing is a function of the temperature. Those kept at 75° to 80° F. metamorphosed in 89 to 109 days, while those kept at 55° to 60° F. took 116 to 120 days. The times required by animals fed on thyroid were always much smaller, varying from 17 to 50 days. The times recorded by Jensen are uniformly less than those found by us. This very probably is connected with a difference of temperature, since our experiments were all carried out in the depth of winter, at temperatures, be it noted, considerably below the lowest used in Boulenger's experiments. Mature animals always require a longer time than do immature. Jensen's results show that the minimum time for metamophosis was 10 days longer in adults than in immature specimens, the maximum 7 days longer; the minimum time for adults was thus greater than the maximum for immature specimens. The difference in our own experiments was still greater. For immature specimens, it does not appear to make any difference whether they are quite young (under 3 inches in length) or half-grown; the time taken is approximately the same. This appears to indicate a real difference between the metabolism of adult and immature specimens, and that the time-difference observed is not due merely to differences of size. We may sum up by saying that thyroid-feeding causes metamorphosis much more rapidly than does enforced air-breathing; and that the change to sexual maturity in Axolotls increases the time necessary for metamorphosis by 25 to 50 per cent.

Some Amblystoma used in our experiments were bred in the laboratory in the spring of 1920. The eggs were laid in the second half of April. Young specimens from these eggs were used for experiment later in the same year. The first experiments were made in the first week of July, the larvæ then being not quite 3 months old, and measuring almost precisely 50 mm. in length. Four of these were fed upon thyroid, and showed all the usual metamorphic changes; the gills were reduced to mere stumps without filaments, the fin along the back was resorbed, and the eyes became prominent. All four, however, died between the fourth and fifth weeks of the thyroid diet, when practically metamorphosed. It was not possible to say whether death was due to internal causes resulting from a too-precocious transformation, or whether it was due to the animals not being able to climb out of the water: that this latter suggestion might well be valid is shown by the fact that the animals' legs at this stage were proportionately smaller, and especially slenderer, than at later stages, and were quite possibly too weak to support the creatures on land. On the other hand, other Amblystoma, transformed by thyroid, usually remained several days in water after completion of all visible morphological metamorphic changes; so that death is most probably to be ascribed to internal changes, especially since it is known that in Anura there is a lower limit of size below which thyroid diet induces an attempt at metamorphosis, but one which is always followed by death.

A single larva was next put on thyroid diet on August 16, 1920, *i.e.*, when about 4 months old and 59 mm. in length. The animal showed metamorphic changes after 15 days, came out of the water after 26 days, and lost the last traces of gills after 29 days. Metamorphosis can thus be induced in Amblystoma that have not yet attained a length of 2½ inches; it is possible from then onwards at any size or age. Three more larvæ were started on the thyroid diet on August 9, 1920, when nearly 5 months old. The first traces of change were observed on the 17th day, the gill-filaments were all resorbed by the 23rd day, and all emerged from the water between the 30th and 32nd days. A slight diminution in size had taken place in the process, the original lengths of 68, 60, and 60 mm. being reduced to 64, 59, and 58 mm. respectively.

The following experiment was carried out to see whether immersion in dilute alcohol would accelerate or retard the metamorphosis caused by thyroid. Five dishes were prepared, one with larva in each. All contained 600 c.c. of liquid. (P) contained water, and the individual in it was fed on meat as a control. (T) also contained water, but the animal in it was fed on thyroid. (Q) contained 1/3 per cent. alcohol, (R) 1/6 per cent. alcohol, and (S) 1/12 per cent. alcohol. The animals in (Q), (R) and (S) were all fed on thyroid. The alcohol solutions were changed thrice weekly. The experiment was started on November 5, 1920. It was very noticeable that the animals in the alcohol solutions were, especially at first, more sluggish than either of those in plain water. They did not at first react to stimulation, but, after the stimulus had continued for some time, they responded by abnormally violent movements. After 2 to 3 weeks, however, they apparently became acclimatised to the alcohol, for their sluggishness was not nearly so marked.

(S) was distinctly less sluggish than the other two, between which not much difference could be noticed.

The experiment was not wholly conclusive. The differences between the rates of metamorphosis of the four thyroid-fed animals was very slight. There was, however, a slight retardation seen in the specimens kept in alcohol, and a retardation which increased with the concentration.

The experiment was therefore repeated with the following variations: (1) Control: meat-fed in water. (2)–(5) Thyroid-fed: (2) in water; (3) in 1/3 per cent. alcohol; (4) in 2/3 per cent. alcohol; (5) in 1 per cent. alcohol; two animals in each dish. The same slight retardation of metamorphosis with alcohol, increasing with concentration, was observed. Again, larger numbers are really necessary before the retardation can be regarded as proved; but, so far as it goes, these experiments appear to be significant.

All the alcohol specimens this time showed redness and congestion of the gills, increasing with concentration. The congested gills were not reduced till relatively very late, and then decreased remarkably rapidly in size. The 2/3 per cent. and 1 per cent. specimens all died suddenly just before they were due to metamorphose. Metamorphosis in the control took 29 days; in the 1/3 per cent. 29 and 31 days.

(d) *Size of Thyroid.*

From all recent work upon the metamorphosis of Amphibia, it would appear certain that metamorphosis is normally associated with thyroid activity, taking place when certain substances produced by the gland reach a definite concentration in the body. In those forms, therefore, in which a typical metamorphosis rarely or never occurs, we should *a priori* expect to find an abnormally small thyroid.

In the Axolotl, however, the thyroid is not abnormally small; further, it presents a perfectly normal histological picture. It would only be possible to give definite data after a careful examination of the relation between thyroid-weight, body-weight, and, if possible, iodine-content (or other criterion of activity) of thyroid in a number of Urodela, including normally metamorphosing forms, the Axolotl, and some Perennibranchiates. There is, however, one observation which it is of interest to mention here. That is the abnormally large size of the thyroid in Siren. This has been recorded by Wilder in his anatomical account of the animal (1891); and his account we have confirmed from the dissection of two specimens preserved in spirit in the Oxford University Museum. The thyroid is not only as large as in a normally-metamorphosing species, but much larger. Of this fact there can only be two explanations. Either the thyroid of Siren is no longer producing the same type of substances as in other Amphibia; or, if it is continuing to do so, the body of the organism does not respond to the substances produced in the same way as in other species. That this latter is a possibility is shown by the example of Proteus and Necturus, which cannot be transformed by thyroid-feeding. In either event, the large size of the thyroid is very peculiar and demands investigation; possibly it has taken on some new function. From the particulars given by Platt (1896) it does not appear that the thyroid is under-developed in Necturus. In Typhlomolge, on the other hand, Emerson (1905) reports the complete absence of a thyroid gland. Leydig (1853) describes the thyroid of Proteus as being small, median, and composed of but 3 to 15 vesicles, which, however, often contained colloid.

(e) *Exophthalmos Associated with Metamorphosis.*

It is an interesting fact, which, so far as we know, has not been previously emphasised, that Amphibia before metamorphosis have their eyes flush with the surface of their head, but that in the adult state the eyes protrude considerably. In the case of the Axolotl the eyes remain flush with the surface so long as the animal remains in the aquatic form, even if it becomes sexually mature. The phenomenon appears to occur equally in Anura and Urodela. In view of the connection of the thyroid with Amphibian metamorphosis, this protrusion of the eyeballs becomes interesting when it is remembered that exophthalmos is one of the most prominent symptoms of Graves's disease. Whether the exophthalmos in Amphibia at metamorphosis has any relation to that of exophthalmic goître cannot be definitely stated.

2. *Acceleration of Metamorphosis by Iodine with Triton and Salamandra Larvæ.*

Following Swingle's results (1919), a solution of iodine was made by shaking up an excess of iodine crystals in tap-water in a 2-litre flask, allowing to stand for 2 days, and then diluting as required with tap-water. Two larvæ of *Salamandra maculosa* were put into each of a series of dishes containing 350 c.c. of fluid each.

No. 1 was the control (tap-water only), the rest contained a saturated solution of iodine, diluted respectively 10, 50, 100, 500, and 1000 times. The experiment was performed at room temperature in January, 1920. In all except the 1/10 solution the animals fed well. In the control and in the 1/100 solution the gill-filaments were unchanged after a week. In the 1/500, the 1/100, and the 1/50 solutions the gill-filaments were somewhat reduced after 5 days. For some reason the greatest reduction occurred in the

1/100 solution. In the 1/10 solution the animals refused to feed at all. One (the smaller) died after one day. The other was more sluggish than normal animals. It showed distinct reduction of the gill-filaments after 3 days. The filaments were nearly absent after 5 days; on this day the solution was diluted to 1/12, as the animal seemed ill at ease. On the eleventh day mere stumps of gills were left, and the tail-fin had been nearly resorbed. The animal was poorly, not reacting to stimuli properly, and on the twelfth day it was dead. The saturated solution contained 0·076 mgrm. of iodine per litre.

Another series of experiments was started on January 26, 1920. Unfortunately, it had to be discontinued after 16 days, owing to the ill-health of Miss F. Peterson, who kindly helped with the work, and to whom grateful acknowledgment is made. Twelve sets of four animals were taken, there being two larvæ of *S. maculosa* and two of *Triton vulgaris* in each lot. These were distributed as follows, according to temperature and to strength of iodine solution:—

Temperature.	Strength of solution (in dilutions of a solution of iodine saturated at room-temperature).			
	1.	2.	3.	4.
A. 26° C.	1/20	1/50	1/125	Control.
B. 17°–18° C.	1/20	1/50	1/125	,,
C. 2°–5° C.	1/20	1/50	1/125	,,

In Series C no animals metamorphosed during the 16 days. In Series B five metamorphosed after an average time of 11·6 days. In Series A five metamorphosed after an average time of 6 days. This shows a very decided retarding effect of low temperature upon metamorphosis.

All animals were here kept in the same volume of water, so that only two variables affecting gill-size remained. High temperature was found to favour gill-development, while high iodine-concentration had the reverse effect. This was well brought out in the results. The gills of those kept at 26° C. could be arranged in a graded series according to size, the controls having the largest filaments, those in the 1/20 solution the smallest. Those kept at 2°–5° C. also showed a series which was identical, except for the fact that it started at a much lower level; in fact, the gills of the controls at the low temperature were slightly smaller on the average than the gills of those kept in the 1/20 solution at the high temperature. The 17°–18° C. series was intermediate, but rather nearer, as would be expected, to the high-temperature series.

Great diversity in the rate of metamorphic change was found, so much so that the original purpose of the experiment—viz., to get some quantitative data on the time-relations of metamorphosis to iodine concentration—had to be given up. It appeared that the prominence of the eyes which accompanies metamorphosis in these forms, might begin at high temperatures while the gills were still hyper-normal in size. Thus the effect of high temperature may be to mask the effect of iodine as far as the gills are concerned. When the resorption of the gills did begin at high temperatures, it went very quickly, the effect of the iodine overcoming the antagonistic effect of temperature. This is probably, according to Swingle, due to the power of all tissues of the body, but especially the thyroid, to manufacture from iodine some substance which causes the initiation of metamorphosis, when it reaches a certain critical concentration inside the body.

It, however, appears probable that the iodine may also have a direct effect on the tissues of the gills. The violent action of the 1/10th solution upon the filaments in the first experiment is very likely to be explained in this way. It is at any rate certain that the filaments are very sensitive to external agencies, as is also the tail-fin. Both of these structures, for instance, are much reduced when Amblystoma larvæ are kept in a very small amount of water or in damp moss, the mechanical alterations seeming to initiate the reduction (although of course the final complete metamorphosis which takes place in these circumstances must depend upon other, more deep-seated changes). We may say, therefore, that low temperature, exposure to air instead of water, and probably iodine solutions, have a directly unfavourable effect upon the gill-filaments, causing a certain amount of dedifferentiation and resorption, while the other agencies, such as thyroid diet and iodine accumulated in the body, exert an indirect effect by altering the internal environment to the point where metamorphic changes are started. Once these begin, the character of the gill-epithelium is altered, and the gills are rapidly reduced to mere blobs (fig. 4).

3. *Negative Effects of Administration of other Ductless Glands and of Iodine on Metamorphosis in Axolotls.*

Attempts were made to induce metamorphosis by administration of iodine in the medium and with the food to three animals 12 cm. in length. In the latter case, as in Swingle's earlier experiments (1919), a small quantity of finely powdered iodine was used, being in this case dusted on to thin slices of meat rolled into pellets. Such treatment does not produce obvious discomfort, nor is it poisonous, and with several animals was continued bi-weekly for 8 to 10 weeks without any diminution of gills and tail-fin or any evident toxic consequences. All our endeavours confirm the conclusion of Jensen that iodine free of organic combination is not efficacious in producing metamorphic changes in the Axolotl. It is thus evident that in the neoteny of the Mexican Salamander it is not primarily the exogenous factors (available iodine supply and temperature) contributing to normal Amphibian metamorphosis that are significant.

Hence it became desirable to test the effect of administering other ductless glands. In this connection the results of D. I. Macht (1919), who has claimed to accelerate transformation in frogs by prostate-feeding, as also Bennet Allen's experiments (1920) on the part played by the pituitary gland (anterior lobe), demanded some attention. On the other hand the experience of Gudernatsch (1914) and others does not indicate the likelihood of influencing Amphibian metamorphosis by administration of pituitary gland *per os.*

Both young (4 to 6 months) and old (18 months) larvæ were fed with pituitary gland (anterior lobe). In the experiments glands of both old animals (ox) and of calves were employed and the treatment was continued for three months without producing metamorphic phenomena. As an illustration, particulars of the following experiment will serve. Fresh ox pituitaries were obtained from a slaughter-house, and the posterior and anterior lobes separated. On October 2, 1920, four vessels were prepared with four larvæ in each, which were nearly 6 months old. Those in vessel A were fed on thyroid; those in B on pituitary anterior lobe (henceforth called pituitary for brevity's sake, since posterior lobe was never employed in any of these experiments); in C on thyroid and pituitary on alternate days; and in D on raw meat, as controls.

Those fed on thyroid showed the first visible signs of transformation on the 14th day, their gill-filaments were resorbed by the 21st day, and the metamorphosis was morphologically complete on the 26th day. The controls showed no change throughout. Those fed on pituitary have showed no metamorphic changes at all; three were kept on the diet for 3 months, one for 8 months. It is obvious that the diet has no effect on their transformation. Those fed alternatively upon pituitary and thyroid metamorphosed in a perfectly normal way; the process, however, took slightly longer, the first signs of change appearing two days later than in those fed only on thyroid, the total resorption of the gill-filaments, and the morphological completion of the process, each taking place 3 days later than with the thyroid-fed ones.

The rates of growth upon the different diets are illuminating; they may best be presented in tabular form :—

	Original length.	Increase in length in 31 days.	
	Average.	Average.	Range.
	mm.	mm.	mm.
A. Thyroid diet	63·0	1·5	0·5–3·0
B. Pituitary diet	62·0	15·0	14·0–16·0
C. Alternate thyroid and pituitary diet	62·5	4·5	3·0–6·5
D. Meat diet	61·5	13·0	10·0–17·0

It will thus be seen that thyroid diet in this experiment did not cause a diminution of size, but permitted a very small increase. The controls fed on meat grew rapidly, but their growth was definitely, if slightly surpassed, by that of the pituitary-fed animals. Those fed alternately on pituitary and thyroid showed only a small amount of growth, but it was almost three times that recorded for those fed on thyroid alone, whereas the proportion of delay of metamorphosis in (C) was by no means equally great. From this, as well as from (B), it would appear that pituitary has definite growth-promoting properties for Amblystoma larvæ.

Half-a-dozen animals used in the pituitary-feeding experiments were taken at intervals of time and transferred to thyroid diet. The time taken for metamorphosis in three cases was slightly longer (33 to 35 days to emergence) than in controls, so that there may have been a retardation. In any case, these larvæ were clearly able to complete their metamorphosis.*

One individual was also fed for 3 months on fresh prostate without showing any perceptible signs of transformation, though subsequent thyroid treatment induced metamorphosis.

4. *Effect of Thyroid-feeding on Necturus.*

The suggestive, but inconclusive, experiments made by Jensen, with a view to elucidating more fully the significance of the Perennibranchiate condition in relation to the physiological processes underlying metamorphosis in other Urodeles, indicated the advisability of extending such observations. It was not possible to obtain specimens of Proteus; but, through the courtesy of Prof. MacBride, three live medium-sized Necturus were secured, and submitted to thyroid treatment in his laboratory at the Imperial College of Science.

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* One animal was continued on the pituitary diet for some time, a control in identical conditions being fed on ox muscle. After five months, the pituitary-fed specimen weighed 32·5 grm., the control 11·2 grm. Other animals of the same age fed on meat were all of about the same size as the control. It has since been suggested to me that if the control had been fed on more succulent diet, such as brain or liver, it would have rivalled the pituitary-fed specimen. Until the suggestion can be tested experimentally, I content myself with stating the facts. The growth of meat-fed specimens in the previous experiment, lasting 31 days, was about as great as that of the pituitary-fed animals. Later, the meat-fed animals' growth became relatively much slower. Another noticeable feature was that in thyroid-metamorphosed specimens previously fed on pituitary the size of the limbs was greater than in those not so treated.—J. S. H.

The experiment began on November 10, 1920. One animal was kept as a control, and fed on small pieces of raw beef: the other two were given fresh thyroid (ox) gland tri-weekly, as in the case of the first experiments with Axolotls. Up till the time of writing, the treatment has been continued without interruption for 7 months. No pigmental changes have resulted; and there has been in neither case any appreciable reduction of the tail-fin. As regards the condition of the external gills, observation is embarrassed by the fact that the filaments are in a very marked degree erectile, their length, when fully dilated with blood, being many times greater than when the animal is not actively respiring: they can, however, be induced to extend by compelling the animal to perform muscular exercise, after which a rough estimate may be made of their maximum dimensions. Constant attention to this point showed very clearly that the filaments of the experimental individuals were relatively shorter even when fully extended; but, for an obvious reason, it is not possible to interpret this as necessarily consequent upon a reduction in the actual amount of tissue; for it is well known that thyroid administration influences the blood pressure in Vertebrates, and the behaviour of the gills in Necturus is evidently a vaso-motor phenomenon.

It does not seem likely, therefore, that an administration of thyroid in this Perennibranchiate form is effective in producing somatic modifications comparable to those occurring at metamorphosis in other Urodeles. In view of the morphological data given by Platt, and of the experimental evidence available, it may therefore be stated that, if the Perennibranchiate forms like Necturus and Siren are *not* primitive in the invariable retention of the larval type of Urodele organisation throughout life, their failure to develop the predominantly adult characteristics is not due primarily to thyroid deficiency—this, of course, does not apply to Typhlomolge, and possibly to Proteus also—nor to a defective supply of iodine in the environment. There remain at least four possible interpretations, then, of the Perennibranchiate state:—

(i) That these animals have never possessed genetic factors responsible for the structures of typical adult Urodela; *i.e.*, that in this respect they are actually primitive. This appears to be negatived by the purely morphological evidence available (see Gadow, 1901, pp. 65, 136).

(ii) That their thyroid mechanism is unable to make use of the available iodine supply so as to produce the requisite amount of active iodine compound required to stimulate metamorphosis.

(iii) That endogenous factors involved in the maintenance of the thyroid in a condition of functional activity are not operating effectively.

(iv) That the larval tissues concerned have collectively lost the power to respond to the thyroid activator.

The second and last possibilities are emphasised by the fact that, whereas in normal Amphibia Swingle has shown that iodine free of organic combination can suffice to induce metamorphosis not only in normal but in thyroidectomised larvæ, in the case of the mature Axolotl, iodine alone is not an efficient substitute for the thyroid autacoid. The importance of the third is sufficiently demonstrated by the work of Uhlenhuth (1919) on the relation of growth to metamorphosis in Salamanders, and Allen's recently published account of the inhibition of transformation in Anura by hypophysectomy. The state of affairs encountered in the Mexican Salamander is eminently suitable to a further analysis of what we have termed the endogenous factors in Amphibian metamorphosis; and it is hoped to obtain shortly in this connection data respecting the relation of the method of thyroid-feeding to enforced air-breathing as a means of bringing about the assumption of adult characters.

5. *Pigment Reactions of Axolotls to Pituitary and Adrenal Hormones.*

While the feeding of Axolotls with pituitary gland did not prove productive of positive data in relation to metamorphosis, it yielded results which encouraged further enquiry into the reaction of Amphibian melanophores to the internal secretions.

A few hours (6–12) after feeding Axolotls on pituitary (whole) gland, a marked darkening of the skin was observed; this was not noticed the first few days of pituitary diet, but became increasingly pronounced as the feeding continued. At first the darkening was very gradual, attaining its maximum intensity about 18 hours after feeding, which took place every 48 hours. Curiously enough, when this effect had quite passed off by the morning of the second day, the animals were of a ghostly pallor—considerably lighter than their normal shade, and remained thus until fed again. The rate of darkening progressively increased; after 3–4 weeks of the pituitary diet the maximum degree of expansion of the pigment cells was reached within an hour. The extreme subsequent paleness still occurred, appearing within 24 hours of feeding. Finally, after about 3 months' treatment the response diminished.

These observations were based on a dozen medium-sized Axolotls (and as many controls) of the albino variety, in which the pigment cells are sparse and confined to the upper surface of the head and mid-dorsal region. The reaction was more pronounced when the whole gland or the posterior lobe alone was administered than when the anterior lobe only was used. While these experiments were in progress Bennet Allen (1920) issued a preliminary notification of experiments on pituitary removal and transplantation in tadpoles, mentioning *inter alia* that animals after pituitary removal display a silvery-white appearance, in marked contrast to the coloration of the normal form. Thus the expansion of melanophores in reaction to the pituitary hormones would not appear to be confined to Axolotls.*

The interest of this reaction is twofold: firstly in relation to Spaeth's thesis (1916) that melanophores represent a modified form of smooth muscle fibre; and secondly, in view of a suggestion by Fuchs (1906) that internal secretion may underlie the well-known phenomenon of "colour adaptation." Spaeth instances, as intermediate in character between typical smooth muscle and pigment cells, the sphincter pupillæ, in which, in certain cases, the cytoplasm is densely charged with melanin granules; he points out that the melanophores are mesodermal in origin; and draws attention to a remarkable parallelism between the reactions of the sphincter pupillæ fibres and melanophores in response to electrical and light stimuli and chemical stimuli such as atropine. To this it may be added that both react in the same way to the pituitary hormone, for it has been shown by Cramer (quoted by Schäfer, 1913) that the latter induces dilatation of the pupil.

The phenomenon of "colour adaptation" in reptiles, amphibia and fishes, has been provocative of much controversy and is little understood. It is widely known that these organisms can respond to the colour of their surroundings by pigmental changes. The most recent work of Laurens (1917) and others shows that, though the melanophores respond directly by expansion in bright light, in the case of Axolotls which have been blinded there is no secondary modification of the response (partial contraction) after continued illumination, and no power to respond to their background, *e.g.*, to become darker when illuminated only from above in a blackened container. The pigmental responses of these animals thus appear to be under the control of stimuli received through the organs of vision. How this control is exerted is at present an unsolved problem.

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* Allen's (and also Smith's) results indicate that it is the intermediate lobe of the pituitary which is concerned in pigmental control in Anura. Presumably the same holds good for the effect of a diet of Mammalian pituitary on the Axolotl. The intermediate lobe will remain attached to the posterior lobe unless specially dissected apart. Swingle's recently published work (1921) confirms and extends the above-mentioned results.

Two possibilities invite consideration. Either the stimuli received by the eyes are transmitted entirely through the nervous system *viâ* the fibres innervating the pigment cells—assuming that in all cases pigmental cells are innervated from the C.N.S.; or nervous stimulation of internal secretions efficient to produce the appropriate reaction may be involved. Obviously both mechanisms may operate concurrently. In order to interpret the pigmental responses in these animals in a manner consonant with the second hypothesis, it is first necessary to demonstrate that the melanophores react in one way to one type of internal secretion and in an opposite sense to another. It has been stated that a pituitary hormone causes the melanophores to expand; and the question arises whether other internal secretions can bring about the reverse effect. In this connection two observations provide a clue. McCord and Allen (1917) have recorded that after feeding tadpoles (*Rana sylvatica*) on pineal glands for 10 days, each subsequent meal was followed by transient, and complete, contraction of melanophores, noticeable in half-an-hour, reaching its maximum in about 45 minutes, and passing off after 2 to 3 hours. Bigney (1919) again finds that by injecting adrenalin into the adult frog, the contraction of the pigment cells is produced, confirming earlier work of Lieben (1906).

To test the reaction of the pigment cells in Axolotls to pineal treatment, eight medium-sized (9 month) larvæ were placed (November, 1920) in separate containers, of which the sides had been blackened, and illuminated from above. In this way maximum expansion of the pigment cells is brought about within a few hours. Four were fed on fresh pineal glands tri-weekly, and the remaining four (controls) were kept on a normal meat diet. The experiment was continued for 2 months and proved quite fruitless. No pigmental differences either of a permanent or temporary character could be observed in Axolotls, although, as will be seen later, McCord and Allen's observations as to the effect of pineal feeding on tadpoles were afterwards confirmed. Two similar experiments with the same numbers were then repeated, with adrenal medulla instead of with pineal gland; whether administered as food or by adding fresh extract to the medium, a complete contraction of the pigment cells invariably ensued, with great vaso-dilatation of the gills.*

It thus appears that the pigment cells of medium-sized Axolotls react in an opposite manner to pituitary and adrenal extracts, and the fact that in Allen's experiments the removal of the pituitary was accomplished by melanophore contraction, is indicative that internal secretion underlies the mechanism by which the regulation of pigmental reactions is effected in normal life. In any case there would appear to exist a double compensating mechanism for the control of the behaviour of the melanophores: though we cannot yet legitimately infer that this constitutes the effective apparatus through which the organs of vision influence them.

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* Probably the effect is in either case due to adrenalin acting on the skin *viâ* the medium.

6. *The Pineal Pigment Cycle in Tadpoles.*

In view of the lack of success with which efforts to induce a response on the part of the melanophores of the Axolotls were attended, it was decided to repeat McCord and F. Allen's experiments upon pineal administration to Anuran tadpoles. About 500 tadpoles of *Rana temporaria* were employed for this purpose in glass containers during the spring of 1921. At first the controls (meat-fed), and the experimental animals which were fed on fresh ox pineal glands tri-weekly, were kept respectively in single containers. Later, for purposes of observation, the tadpoles were separated in glass bowls (placed on a white background) in colonies of twenty. Contemporaneously, tadpoles raised from eggs laid on the same day were being fed on suprarenal cortex, suprarenal medulla, corpus luteum, and anterior pituitary lobe for ulterior purposes, so that it was possible to compare the phenomena consequent upon pineal treatment with the results of administration of a more varied range of tissue extracts than were employed by the authors named above.

In these experiments no change in the pigmental characteristics of the tadpoles was noticed during the first fortnight of pineal diet, which began about a fortnight after hatching. Before three weeks had elapsed, the phenomena of the pineal pigment cycle, as recorded by its discoverers, became evident. Within a quarter of an hour of feeding the tadpoles became visibly more pale, till, when the reaction reached its maximum, half an hour after the meal commenced, they assumed a quite unique appearance, by virtue of the contrast between the complete translucence of the head region and tail on the one hand, and the opacity of the visceral portion of the body on the other. This condition passes off after the lapse of five or six hours: till metamorphosis took place the same reaction followed each administration with the utmost regularity. No modification of the pigmental features occurred in the controls or in the additional cultures which were being fed synchronously with other glandular tissues. Histological preparations confirmed McCord and Allen's conclusion that the behaviour of the melanophores is the significant element in the situation. After a pineal meal the melanophores are fully contracted, the pigment being congregated into compact masses in the centre of the cell.

The time relations observed are somewhat different in these experiments from those obtained by McCord and Allen. Thus the latent period was a little longer; the time required for the effect to manifest itself after feeding a little shorter, and the duration of the effect considerably longer. In the case of *R. sylvatica*, these investigators found that the reaction passes off in about two hours. Apart from these insignificant details, the experiment recorded entirely confirms their results. It may be added that extracts were also employed and produced rapidly (less) corresponding effects to those produced by administration *per os.*

As regards the cortex-fed cultures, no difference was seen in the pigmentation of tadpoles fed continuously from hatching till metamorphosis on suprarenal cortex as contrasted with controls. We take the opportunity of putting this observation on record, because Gudernatsch (1914) stated that cortex-fed tadpoles show progressively less pigment after five weeks' treatment, an observation which, if confirmed, would seem significant in relation to the ætiology of Addison's disease.

That the pineal gland of Amphibia does actually function in relation to pigmental responses, although it is probable, cannot be legitimately contended on the basis of evidence so far available, as mammalian pineal glands were employed in the experiments. What can be definitely stated is that pineal tissue is specifically distinguished by the possession of a physiologically active substance, a conclusion which goes far to establish its claim to be classified as an endocrine organ.

Summary.

A. *Metamorphosis.*

1. Salamandra and Triton larvæ may be metamorphosed by immersion in a dilute solution of iodine. Metamorphosis is retarded by low temperature. High temperature at first causes increased growth of the gills.

2. Sexually mature Axolotls can, as Laufberger and Jensen originally showed, be made to undergo metamorphosis by means of a thyroid diet.

3. Metamorphosis is accompanied by exophthalmos, apparently in all Amphibia.

4. In the case of the Axolotl, the time required for metamorphosis induced by enforced air-breathing is considerably longer than when induced by thyroid-feeding; in the latter case, it is longer for sexually mature than for young larvæ; and is in all cases accelerated by increase of temperature.

5. Administration of iodine free of organic combination, or fresh glandular

substance of the prostate and pituitary anterior lobe, is without any effect in relation to the metamorphosis of the Axolotl.

6. Thyroid-feeding continued for 7 months was not accompanied by any noteworthy somatic changes in Necturus.

B. *Pigmental Responses.*

7. Pituitary feeding (posterior lobe or whole gland) produces a marked temporary dilatation, followed later by excessive contraction, of the dermal melanophores in albino Axolotls.

8. Adrenal medulla extract produces temporarily complete contraction of the dermal melanophores in the Axolotl.

9. Pineal administration (as extract or as food) rapidly brings about a striking transient contraction of the dermal melanophores in frog tadpoles: McCord and F. Allen's observations in this connection are fully confirmed. It has, however, no effect upon the melanophores of the Axolotl.

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Studies in Bacterial Variability.—On the Occurrence and Development of Dys-agglutinable, Eu-agglutinable and Hyper-agglutinable Forms of Certain Bacteria. (A Report to the Medical Research Council.)

By E. W. Ainley Walker.

(Communicated by Prof. Georges Dreyer, F.R.S. Received October 12, 1921.)

(From the Department of Pathology, University of Oxford.)

Introductory.

That different cultures of an agglutinable bacterium may exhibit wide differences in relative agglutinability when tested with the same agglutinating serum is a familiar fact. But the conditions on which these differences depend remain to a great extent obscure. Yet a number of facts which bear upon the problem have come to light in the course of investigations carried out by various observers.

A good many years ago the present writer showed (1901) (1) that if a series of strains* of *B. typhosus* be employed in preparing a corresponding series of agglutinating serums, each such serum is found to act more powerfully upon its homologous culture than upon any of the heterologous strains. It was, therefore, stated that the serums were not only specific for the species of bacterium in question, but also *special* in each case to the particular strain employed in its production. It also appeared that, so far as the evidence went, the heterologous strains always fell into the same order of relative agglutinability when tested with the different "special" serums.

Somewhat similar results have recently been published by A. D. Gardner (1920) (2), in connection with his investigation of Paratyphoid A. serums, and he has shown that the peculiar sensitiveness of a given strain to agglutination by serum prepared with that particular strain, which I had indicated by the term "special" is, in part at least, a matter of velocity of reaction. The phenomenon (so far as it depends on reaction rate) was, therefore, spoken of by him as a "super-specific acceleration" of reaction.

Since my own experiments were made before the introduction of the present accurately standardised methods of determining agglutination titre, I had re-examined the question in another relation (1918) (3) with four strains of *B. typhosus.* A rabbit was immunised successively with three of the strains, and on each occasion the titre of its serum rose to a higher level for the strain used as antigen on that occasion, than for either of the other three strains. Furthermore the four strains were seen to belong, two and two, to two serological groups which were widely differentiated from each other by their relative sensitiveness to agglutination by different serums.*

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* In the use of the terms "strains" nothing is implied with regard to the cultures so spoken of save that they have been obtained from different sources, derived by different methods of cultivation, or selected in any way for a particular purpose. If they show differences, it is a matter for investigation in each case, whether such differences are permanent or fleeting.

Somewhat similar serological differences are now well known in certain other organisations, *e.g.*, Meningococcus, Pneumococcus; and they may at times be very marked indeed, so that particular strains are described as "inagglutinable"—for example inagglutinable strains of *B. typhosus.* Yet it is found that on sub-culture these inagglutinable strains will sooner or later yield cultures which give good agglutination. Thus the inagglutinability appears to be only a phase, or temporary character. The inagglutinable form of the organism *cannot, therefore, on the existing evidence be regarded as mutant,* in the proper sense of the term; but it presents an example of bacterial *fluctuation.*

Since the observations just referred to (1918) (3) raised a number of interesting questions the subject has been re-examined in detail during the progress of the present investigation by A. D. Gardner and Ainley Walker (1921) (4) who obtained inagglutinable strains of *B. typhosus,* and compared them serologically with ordinary "good agglutinators." The existence of the two serologically different types of *B. typhosus* was fully confirmed. It was shown that they corresponded to a motile and a non-motile phase respectively of the bacillus. And it was further shown that *from a single culture* of the bacillus colonies could on occasion be isolated by plating which would on cultivation give rise to populations differing as widely, both serologically and as regards motility, as any strains obtained from different sources.

The serological difference between the agglutinable (motile) form and the inagglutinable (non-motile) form of the bacillus was an antigenic difference. For if a serum were prepared with the inagglutinable form, it agglutinated its own bacillus and other inagglutinable strains quite well, and to high titre. But in contrast with the rapidly-formed, large and fluffy flocculi produced in an ordinarily well-agglutinating culture, the clumps produced in inagglutinable suspensions are always small, compact and slowly formed.

On this account, and in view of what is to follow, I propose the term *dys-agglutinable*† as a more appropriate designation than "inagglutinable" for this phase of the bacillus. The ordinarily well-agglutinable phase may then be spoken of as *eu-agglutinable* (where distinction is necessary) and the term *hyper-agglutinable* employed for phases where agglutinability is found to be considerably increased. The differences in agglutinability, and the antigenic differences in strains from different sources, and in sub-cultures from different colonies studied by Gardner and myself arose without experimental interference; that is to say they were naturally occurring differences. So also appear to be the differences recently described in *B. dysenteriæ* (Shiga and Flexner—Y), and several other organisms including *B. typhosus*, in an important paper by Arkwright (1921) (5). Vines also has described and studied strains of meningococcus "hypersensitive" to agglutination (1918) (6) as naturally occurring types. But the results to be recorded show that it is possible to produce experimentally similar differences of agglutinability in many varities of bacteria.

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* A more complete record of this experiment was published by Gardner and Ainley Walker (1921) (4).

† This is a hybrid word; but so are a good number of other accepted and useful terms.

In the course of experiment carried out in 1901–2, I found that the agglutinability of *B. typhosus* was lessened by growing it in a succession of sub-cultures in typhoid agglutinating serum diluted with ordinary culture bouillon (1902) (7). This diminution of agglutinability, and certain other phenomena observed, were interpreted in terms of Erlich's theory, with the conceptions of which I was then imbued, and that interpretation needs reconsideration and may require revision in the light of present knowledge. For it is evident that a diminution in the agglutinability of an organism occurring under these conditions is open to more than one possible explanation, since the production of a relatively inagglutinable race of bacilli might be brought about in several different ways.

Thus it might be due (1) to the gradual training and education of the *whole mass of the population* in successive generations to resist the action of agglutinins; for example by the formation of anti-agglutinins, as I formerly concluded; (2) or it might be due to a selective encouragement of the *propagation of the less agglutinable* individuals (in a population composed of elements differing widely in agglutinability) by some cause facilitating their multiplication in successive generations, to the gradual exclusion and elimination of the more agglutinable individuals; (3) or both processes might play a part.

The first process would offer an example of the heightening and development of a selected character, originally latent, but more or less common to the individuals composing the bacterial population; the second, one of the selective propagation, from a population whose members differed widely among themselves in this respect, of particular individuals in which that character was *already* highly developed. Put briefly, the one would be the *selection of a character* for artificial increase, the other the *selection of individuals* for multiplication. And it is obvious that both processes might be at work side by side.

In view of these considerations, it seemed likely that information of interest would be obtained by inquiring what degree of parallelism, if any, existed between the differences in agglutinability to be observed in different strains (however derived), and those obtainable by repeated cultivation in agglutinating serum. In particular it was hoped to throw some light on the nature and origin of so-called "serological strains" in different species of bacteria. Accordingly experiments have been carried out as described below. Meanwhile the experiments of Gardner and myself already referred to (*loc. cit.*) have shown that extremely wide differences in agglutinability, such as might well form a basis for either process of selection, do actually exist between the individuals which constitute the population of a single culture.

In the account which follows, the experimental work has been summarised so far as possible, and detailed data are recorded only for *B. typhosus*, in order to save space. A general survey of the phenomena under investigation is given, but only a selection of the experiments is recorded in illustration. In certain directions the results are preliminary, and a number of points remain to be worked out in detail.

The Experimental Production of Dys-agglutinable and Hyper-agglutinable Strains (*or Phases*) *of Bacteria.*

By repeated subculture in specific agglutinating serums diluted appropriately with culture bouillon, *dys-agglutinable* forms have been obtained of the following bacteria, ten in all, which were all taken from strains then in use in the department: *B. typhosus*, *B. paratyphosus A.*, *B. paratyphosus B.*, *B. œrtrycke*, *B. enteritidis* (Gaertner), *B. coli*, *B. dysenteriæ* (Shiga), *B. dysenteriæ* (Flexner), V, W and Z.

On transference from the serum bouillon to ordinary culture bouillon, these dys-agglutinable forms yield cultures which agglutinate with difficulty, if at all, with corresponding ordinary agglutinating serums; and those of them that were originally motile organisms are found to have passed into a very feebly motile or entirely non-motile phase. After formolisation and dilution to the opacity of standardised agglutinable cultures, they yielded good and uniform suspensions for agglutination tests, though in a number of cases this result was not obtained immediately or very easily. The dys-agglutinable phase of *B. typhosus* and of *B. dysenteriæ* (Flexner), V, W and Z readily yielded excellent suspensions on a number of occasions.

In other cases difficulty sometimes occurred for one of two reasons—

firstly, that in the case of the motile bacilli, the non-motile phase, when first obtained, often shows a tendency to revert very quickly to the motile eu-agglutinable phase in bouillon culture; and, secondly, because, when rapid reversion does not occur, organisms in this group, and also in the dysentery group, which have been grown in a succession of cultures in dilutions of the homologous agglutinating serum, most often grow in early bouillon subcultures in the form of a deposit with clear supernatant fluid. Although these deposits may often shake up to form perfectly good suspensions for formolisation and dilution to standard opacity, they yield on other occasions suspensions in which many of the bacilli remain more or less agglomerated. The latter suspensions are always undesirable for use in any kind of observations on serum agglutination, and they were put aside as quite unsuitable for experimentation, if, after the usual period of heating in the water-bath, followed by 24 hours' standing at room temperature, there was any deposit whatever at the foot of the control tube, or more than a faint granulation to be seen (with a lens) in the fluid.

B. typhosus.

An actively motile agglutinable bouillon culture of *B. typhosus* (T.e.)* was taken from a strain then in use, and a stock of formolised agglutinable culture of standardised opacity was prepared from it. A subculture was preserved in agar stab, and a subculture was made in the agglutinating serum of rabbits immunised with T.e., diluted 1 to 9 bouillon. The latter culture grew in the form of flocculi of agglutinated bacilli, which sank to the foot of the tube. It was carried on at intervals of 48 hours in small tubes in a succession of cultures in the diluted serum through nine passages. The serum dilutions were tested for sterility before use by incubation for 48 hours at 37° C.

The successive subcultures were always made from the top of the fluid, care being taken not to disturb the deposit. In the first and second cultures this fluid looked clear; but, in the later cultures, an increasing turbidity of the fluid appeared, in addition to the flocculent deposit at the foot of the tube. This appearance suggested that an increasing number of the bacilli present were insusceptible to the agglutinating action of the serum bouillon medium. Nevertheless, the microscope often showed the presence of small clumps among the free bacilli.

From the fifth and ninth serum bouillon culture ordinary bouillon cultures were made, and, from these, subcultures (T.e. 5 and T.e. 9) were prepared

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* The same strain as that denoted T (E) in the paper by Gardner and myself already referred to (4).

24 hours later in 250-c.c. flasks containing 100 c.c. of bouillon. The latter were grown for 24 hours at 37° C., and then well shaken up and formolised. They were placed in cold storage and shaken up several times a day for 3 or 4 days, and were subsequently diluted with formolised normal saline solution to standard opacity. Microscopic examination before formolisation showed that T.e. 5 was almost entirely in the non-motile phase, T.e. 9 short and very highly motile. Serums were prepared by inoculating rabbits intravenously with 1 c.c. of these formolised cultures, and taking blood on the eighth day. Rabbit 1 received 1 c.c. of culture T.e. and serum was prepared (serum I, 1); a month later it received 1 c.c. of culture, T.e. 5, yielding serum I, 2, and, after the lapse of a month more, it received 1 c.c. of T.e. 9 and yielded serum I, 3.

On the same day as the last of these inoculations, rabbits 2 and 3 received intravenous inoculations of 1 c.c. of cultures T.e. 5 and T.e. 9 respectively, and serums II and III were subsequently obtained from them by bleeding on the usual day (eighth).

These serums, together with a 100-unit standard serum (Dreyer), were then tested out against the cultures T.e., T.e. 5 and T.e. 9, using the series of dilutions 1 in 25, 50, 125, 250, etc., of Dreyer's series, and recording the results in Dreyer's notation of total (t), standard (s), trace (tr), and intermediate values (*e.g.*, s −, tr +) as required. The tubes were read after 2 hours in the water-bath at 54° to 56° C., followed by 15 minutes' standing at room temperature, and again after 24 hours at room temperature.

Table I.—Showing Dilutions of Serums yielding "Trace" Agglutination.

Culture.	2 hours' readings.				24 hours' readings.			
	T. 36	T.e.	T.e. 5.	T.e. 9.	T. 36.	T.e.	T.e. 5.	T.e. 9.
100-unit Standard serum...	750	800	10	1,200	1,200	1,000	900	2,500
Serum I, 1	—	1,600	20	2,500	—	1,900	800	4,000
Serum I, 2	—	5,000	450	4,500	—	6,000	1,900	5,500
Serum I, 3	—	4,500	450	4,500	—	6,000	1,900	9,000
Serum II	900	1,900	100	2,200	1,300	2,200	2,000	2,600
Serum III	18,000	10,000	20	18,000	20,000	20,000	175	45,000

Serum I, 1 from Rabbit 1, inoculated with 1 c.c. T.e.
" I, 2 " 1, " " 1 c.c. T.e. 5, a month later.
" I, 3 " 1, " " 1 c.c. T.e. 9, a month later still.
" II " 2, , " 1 c.c. T.e. 5.
" III " 3, " " 1 c.c. T.e. 9.

The complete record of these tests is omitted from considerations of space. But the end-point readings, after 2 hours and after 24 hours, are presented numerically in the subjoined Table (Table I), where the figure given represents the dilution of the serum observed for a reading of trace (tr), or estimated from an observed end-point reading of trace plus (tr+) or trace minus (tr−).

From this Table, and in connection with it, the following points emerge:—

1. In the culture T.e. 5 the bacillus has become distinctly dys-agglutinable. The 2 hours' readings show a very low agglutination titre even in the three serums prepared with this culture itself, either alone (serum II) or following a previous inoculation of T.e. (serums I, 2 and I, 3).

In the 24 hours' readings the end-point titre has advanced to a fair height, except in the case of serum III. But it is to be stated in this connection that, in the tests made on this culture (T.e. 5), *no tube in any series showed a reading higher than trace plus* (tr+), even when as many as six or seven tubes in series all showed some agglutination, save in the case of the two serums I, 2 and I, 3, where, after two and three inoculations respectively, the serum of rabbit 1 gave marked agglutination (total or standard) in the first two dilutions (1 in 25 and 1 in 50) after 24 hours. Even in these cases the agglutination was of the "dysenteric" type, described by Gardner and myself as characteristic of so-called "inagglutinable" strains of *B. typhosus.* That is to say, the clumps were small, compact, and slowly formed, in contrast with the rapidly formed, large and fluffy flocculi of ordinary typhoid agglutination.

2. The culture T.e. 9, obtained by sampling the supernatant fluid of the ninth successive subculture in serum bouillon, is obviously not at all dys-agglutinable. On the contrary, it is much more agglutinable than the original T.e. It would seem to follow that, at this stage at any rate, the change produced by growth in diluted agglutinating serum *cannot be one of progressive diminution of agglutinability in the whole population.*

It is rather a mechanical separation (by clumping and sedimentation) of the more agglutinable individuals from the less agglutinable, probably accompanied by facilitation of the growth and multiplication of the latter, particularly in so far as they remain unclumped.

But since, as already mentioned, the supernatant fluid often contains (microscopically) many small undeposited clumps, it might easily happen, and presumably did happen in the case of culture T.e. 9, that the sample taken for subcultivation in ordinary bouillon contained enough (clumped) motile agglutinable bacilli rapidly to outgrow the dys-agglutinable non-motile individuals in the course of two successive subcultures in bouillon, the second of which constituted culture T.e. 9. This point is further illustrated in a later experiment.

So far there had been obtained from the culture T.e. cultures T.e. 5 and T.e. 9, the former definitely dys-agglutinable,. the latter a good deal more agglutinable than T.e., in fact, two and a half times as agglutinable when referred to standard serum.

3. Culture T.e. 9 produced in rabbit 3 a serum of very high agglutinating power for T.e. and T.e. 9. This, however, does not indicate that in T.e. 9 one has isolated a specially powerful antigenic form of the bacillus. It only shows that rabbit 3 was a particularly good subject for the production of agglutinins. For on looking at the serums obtained from rabbit 1, it is seen that T.e. 9, which was used for the third inoculation, did not in this animal cause any increase of T.e. agglutination above its previous level.

On the other hand, it is very evident that T.e. 9 was remarkably poor in antigenic power for the dys-agglutinable bacillus T.e. 5. For whereas serum III (T.e. 9) was nine or ten times as powerful (24 hours readings) in its action on T.e. 9 as serum I, 1 (T.e.), it was only about one-fifth as strong against T.e. 5. That is to say that in culture T.e. 9 the antigenic power of the original T.e. has been *reduced* to about one-fiftieth as concerns T.e. 5.

4. Serum II (T.e. 5) is seen to be much more powerful in its action on T.e. 5 culture than either of the other serums from single inoculations, and as powerful (24 hours readings) as the serums I, 2 and I, 3, though these serums act two or three times as strongly against cultures T.e. and T.e. 9 as does serum II. Nevertheless, T.e. 5, which has become markedly dys-agglutinable, still retains at this stage good antigenic power in forming agglutinins for T.e.

There is, however, some evidence that its antigenic power for T.e. has undergone reduction, since though serum II (T.e. 5) is definitely stronger than serum I, 1 (T.e.) for the original culture T.e. (say 16 per cent.), it was perceptibly weaker in its action on culture T.e. 9 (say 7 per cent.).

In view of the foregoing considerations, it would seem that by growing the bacillus T.e. in agglutinating serum one was in process of separating out, whether by the mechanical action of clamping and sedimentation alone, or by this combined with an influence favourable to the preferential multiplication of the less agglutinable members of the population, a dys-agglutinable form of the bacillus; and that incidentally one also isolated a particularly highly agglutinable form, T.e. 9.

In this latter culture dys-agglutinable elements were much less well represented than in the original T.e. from which both had been derived, as shown by its feeble antigenic action in relation to culture T.e. 5, when used for the production of agglutinating serum. But that they were by no means absent is rendered probable by an interesting observation which now requires mention, namely, that in the numerous agglutination tests made with T.e. 9 culture a faint haze of unagglutinated bacilli always remained in the supernatant fluid of tubes that would otherwise have been recorded as exhibiting "total" agglutination owing to the complete deposition of the large fluffy flocculi of agglutinated bacilli.

It is of interest to note that, even at this comparatively early stage of differentiation both serum II (T.e. 5) and serum III (T.e. 9) act more strongly on their own homologous culture than upon the standard culture T. 36, though this derived itself (at a much earlier date) from the same original source as culture T.e.

Further investigation of the facts observed was attempted by making a similar experiment in duplicate (in 1 to 3 dilution of serum bouillon) and endeavouring to lead cultures of the bacillus along two diverging paths by making each subculture in one series of tubes from the top of the fluid in the preceding culture, and in the other series making each subculture from the deposit of agglutinated bacilli at the foot of the preceding tube.

The experiment was carried on in both directions through nine successive subcultures (18 days) in serum bouillon. Cultures in ordinary bouillon were made from both series at each stage and formolised after 24 hours' incubation, in the hope of obtaining in the one series an increasingly dys-agglutinable culture, and in the other one increasingly hyper-agglutinable.

But the experiment failed in this respect, owing to the fact (which it revealed) that not only did the supernatant fluid of these serum-bouillon cultures often contain a good many microscopic clumps of agglutinated bacilli, but also the deposit held many unagglutinated (dys-agglutinable) bacilli in the interstices of the flocculent mass. Thus the results yielded cultures no more advanced in the desired directions respectively than T.e. 5 and T.e. 9.

The question of how far the effect of growth in agglutinating serum might consist in the mere mechanical separation of pre-existing agglutinable and dys-agglutinable elements in a given culture was next considered. It was found that it is *sometimes possible* to obtain a dys-agglutinable non-motile culture of *B. typhosus* at the first attempt by simply putting up, with due precautions, a 1-in-10 agglutination test with living culture T.e. in a plugged sterile centrifuge tube, incubating at 37° C. until agglutination was well advanced, and then centrifugalising down the agglutinated bacteria, and making a culture or plating from the supernatant fluid.

From this it follows that, at any rate in a considerable number of cases, there must be numerous dys-agglutinable individuals present in an ordinary bouillon culture of *B. typhosus.*

Moreover, when this method fails to give a dys-agglutinable bacillus, as may occur either because dys-agglutinable individuals are relatively few in number in the original culture (or possibly absent), or because they rapidly revert on cultivation in bouillon, success may often be obtained by combining the method of successive cultures in serum-bouillon with severe centrifugalisation of each culture to deposit all clumps before proceeding to the next subcultivation.

In another experiment, the bacillus T.e. was grown in media containing agglutinating serum for a period of four months. The successive subcultures were made at first every second day, then once a week, and later still at intervals of two or three weeks. At the end of this period a subculture was made in ordinary bouillon. On microscopical examination, after 24 hours' incubation the bacilli were quite non-motile. Many fields were examined and no motile individual seen. From this bouillon culture (A) platings were made on a set of agar plates (*vide infra*), and a flask containing a litre of bouillon was inoculated and incubated for 24 hours. The resulting growth (T. dys.) consisted entirely of non-motile bacilli so far as could be seen, and after formolisation and dilution to standard opacity it was found to be very highly dys-agglutinable. It showed characters similar to those described by Gardner and myself (*loc. cit.* (4)) in our so-called "inagglutinable" cultures, and gave *no agglutination* whatever with 100-unit standard serum even in 1 in 25 dilution after 2 hours in the water-bath.

It also *failed to absorb* any appreciable quantity of agglutinins from standard serum. Thus two portions of a particular standard typhoid serum were taken and diluted 1 in 10 the one (*a*) with standardised agglutinable culture T. 36, the other (*b*) with the formolised culture T. dys. The tubes were kept in the water-bath at 54°–56° C. for 4 hours, and subsequently at the room temperature for 24 hours. In tube (*a*), T. 36 was totally agglutinated when first examined at the end of 2 hours. In tube (*b*), T. dys. showed no agglutination after 2 hours, but a trace (tr) at the end of the 4 hours in the water-bath, and 24 hours later it showed a weak standard (s—) agglutination.

Both tubes were then centrifugalised, and the supernatant fluid removed and denoted serum (*a*) and serum (*b*) respectively, and tested along with the original standard serum on culture T. 36 with the results shown in detail in Table II.

The sample of serum absorbed with T. 36 is seen to have been reduced to something between one-fourth and one-fifth of its original agglutinating power for T. 36; whereas that absorbed with T. dys. shows no measurable loss at all of agglutinins for T. 36, but on the contrary the appearance of a *slight increase.* That is to say T. dys. was not only highly dys-agglutinable, but it was also incapable of absorbing appreciably ordinary typhoid agglutinins.

Table II.

Culture T. 36.	Readings at:	25	50	125	250	500	1000	2500	5000	Controls.
Standard serum	2 hours	t	t	t	t−	s−	tr	0	0	0
	24 „	t	t	t	t−	s+	tr	0	0	0
Serum (*a*)	2 „	t	s+	tr	tr−	0	0	0	0	0
	24 „	t	t	tr+	tr−	0	0	0	0	0
Serum (*b*)	2 „	t	t	t	t−	s	tr	0	0	0
	24 „	t	t	t	t	t−	s−	0	0	0

Standard serum 100-unit standardised serum (Dreyer).
Serum (*a*)—Standard serum absorbed with T. 36.
Serum (*b*)— „ „ „ T. dys.

It is important to add that T. dys. was subsequently brought back to the eu-agglutinating phase. After a long residence in bouillon with numerous subcultures its lineal descendants were found to have reverted to the motile phase, and gave good agglutination with standard typhoid serum. A very similar result was subsequently obtained with a highly dys-agglutinable culture of *B. paratyphosus B.*

It is clear that observations of this character must profoundly modify our views regarding the meaning and importance of serological differences, and the results obtained by absorption methods, particularly when such differences are only quantitative and not qualitative. They suggest that "serological strains" of bacteria, even when apparently permanent, may represent no more than particular phases of activity of the bacterial type concerned. As to how such phasic differences may arise we have at present little evidence. How they are maintained for long periods through successive generations is a problem that urgently demands investigation. But it must certainly be admitted as conceivable that changes which can be induced in test-tube experiments, may on occasion also be produced in the body of an infected individual. *If growth in the presence of agglutinating serum under laboratory conditions can lead to serological changes in one or other direction, it must be accepted as possible that in the living body the agglutinin-containing fluids and agglutinin-producing tissues of the animal may also under suitable conditions exert similar influences.* In this connection the occasional isolation of "inagglutinable" typhoid bacilli from cases of typhoid fever, and the serological diversity of the strains of dysentery (Flexner) isolated during recent epidemics of dysentery afford suggestive evidence.

The bouillon culture (A) of which T. dys. was an immediate subculture had been plated out on agar as already mentioned. From these plates 20 colonies were picked off into bouillon on each of the succeeding 3 days. They were incubated for 24 hours, and then examined for motility and formolised for agglutination tests. All these 60 cultures were highly dys-agglutinable, and they were all non-motile, with the exception of one out of the third set of 20. This culture which, it will be noted, had grown for 5 days on serum-free media and had been three times subcultivated (once on agar and twice in bouillon), *had begun to revert,* and showed a certain proportion (possibly as high as 20 per cent.) of motile individuals among the mass of non-motile elements. It is interesting to observe that this proportion of motile elements was not sufficient to render the culture eu-agglutinable.

So far, then, as the evidence from the bouillon subcultures of sixty colonies goes, the original T. dys. culture consisted entirely of non-motile bacilli.

It is not necessary on the present occasion to go into much detail in describing results which showed *increase of agglutinability and the occurrence of hyper-agglutinable forms.*

The culture T.e. 9 exhibited a degree of hyper-agglutinability. It was about two and a half times as agglutinable by standard serum as T.e. or T. 36. Furthermore, the culture T. 36, with which it was compared, was itself a highly agglutinable culture. It had an index of 7, that is to say, it was nearly three times as agglutinable as the original standard agglutinable culture on which Dreyer's unit was chosen, whose index was 2·5. If, as I suggest, this original standard be taken as the mean standard of eu-agglutinability, the culture T.e. 9 was seven times as agglutinable as that standard, and may be regarded as distinctly hyper-agglutinable. In further experiments, a still more highly hyper-agglutinable culture (about twice as agglutinable as T.e. 9) was readily obtained by growing the bacillus in serum bouillon, centrifugalising out the flocculent clumps at an early stage, and making the succeeding serum bouillon culture from the deposit, and so on, instead of working from the supernatant fluid, as in the pursuit of dys-agglutinable forms.

Similarly, starting from a dys-agglutinable culture, one can frequently restore it to eu-agglutinability by the same procedure. Needless to say, all these operations demand rigorous precautions against accidental contamination of the cultures. The method has a practical application which deserves mention.

Strains are sometimes met with in the case of *B. typhosus* (and the same is true of a number of other bacteria), which are "bad agglutinators." Or it may be that a strain, which for a period (possibly for years) has yielded excellent eu-agglutinable cultures, begins, for totally unknown reasons, to give bad agglutinators. Sometimes these "bad" cultures are really more or less dys-agglutinable. They probably contain a fairly large proportion of dys-agglutinable individuals, so that, in a series of agglutination tubes, a haze of opacity remains in those which should show total agglutination, and the series tails off in a succession of "traces" instead of ending sharply. Such a culture cannot be used for standardisation. At other times they are self-agglomerated (self-agglutinated, auto-agglutinated) growths, which may refuse to shake up into uniform suspensions. These difficulties can usually be overcome by repeated daily subculture in bouillon for 14 days or more, as recommended by Dreyer. But that plan does not always succeed. In such cases a eu-agglutinable culture can often be obtained without much difficulty by the methods already described, working from the deposits if the culture was dys-agglutinable, or from the supernatant fluids if it was in a self-agglomerating phase. In confirmation, it may be stated that Dr. A. D. Gardner, to whom I communicated the method, informs me that on one occasion it enabled him to obtain a good agglutinator from an otherwise intractable culture. A similar separation can also *sometimes* be obtained by mere plating and selection of colonies.

B. paratyphosus A., *B. paratyphosus B.*, *B. œrtrycke B.*, *B. enteritidis* (Gaertner), *B. coli*, *B. dysenteriæ* (Shiga), *B. dysenteriæ* (Flexner), V, W, X, Y, Z, *Vib. choleræ.*

All these organisms were tried during the summer and autumn of 1920, at the same time as the earlier experiments with *B. typhosus*, by the method of successive subcultures in diluted agglutinating serums. A dys-agglutinable culture of *B. paratyphosus A.* was readily obtained; one of *B. paratyphosus B.*, with greater difficulty, owing chiefly to a strong tendency to revert when brought into ordinary bouillon culture. *B. coli* also yielded a dys-agglutinable non-motile phase. *B. œrtrycke* and *B. enteritidis* (Gaertner), were found very difficult to manage.

On one occasion only, in each case, was a non-motile dys-agglutinable bouillon culture obtained in a test-tube. Massive subculture in flask seemed to lead to immediate reversion to the motile eu-agglutinable phase, and some of the cultures were hyper-agglutinable as compared with standard cultures when tested with standard serum. In another attempt, made with the same strain of *B. enteritidis* (Gaertner), during 6 weeks of this year (1921), though non-motile or largely non-motile colonies were several times obtained on agar plates from serum bouillon cultures, transference to bouillon invariably led to immediate reversion.

Vib. choleræ.—No dys-agglutinable phase has been obtained at present in this vibrio.

All the foregoing organisms show a tendency to the *formation of a pellicle* in serum-bouillon culture. *B. typhosus*, occasionally only and very delicate; *B. paratyphosus* A and B, and *B. coli*, fairly often and well-marked; *Vib. choleræ*, frequently; *B. œrtrycke* and *B. enteritidis* (Gaertner), very frequent and very heavy, often appearing to contain as much growth in the pellicle as in the deposit at the foot of the tube after 48 hours. These statements apply to cultures made in narrow agglutination tubes.

B. dysenteriæ (Shiga) yielded a dys-agglutinable culture after eleven passages in serum-bouillon of 1 in 4 dilution.

B. dysenteriæ (Flexner), V, W, X, Y, Z.—These five strains of Flexner dysentery gave interesting results after six passages through 1 in 4 bouillon dilutions of their appropriate serums. Bouillon cultures were made in flasks from the sixth passages, and subsequently formolised and diluted to standard opacity. V, W, and Z cultures were dys-agglutinable; X was not perceptibly altered; and the Y culture possessed markedly increased agglutinability, being four times as agglutinable as the standard culture against which it was tested. Of the three dys-agglutinable cultures, Z gave no agglutination at all in 1 in 25 standard Z serum at the end of 4 hours in the water-bath, and no more than a trace minus at the end of the subsequent 24 hours at room temperature. W gave trace minus (tr −) at 1 in 25 after 4 hours, trace plus (tr +) at 1 in 25 and trace (tr) at 1 in 50, 24 hours later. V, on the other hand, though giving no reading as high as trace plus (tr +) in any dilution, showed traces of agglutination in every tube up to 1 in 1000, thus reaching the same actual end-point with its "traces" as did the corresponding standard V culture, which gave totals up to 1 in 250, standard (s) at 500, and trace minus (tr −) at 1000.

The discussion of the relation of these dys-agglutinable and well-agglutinable forms of bacteria to the R and S forms recently described by Arkwright (*loc. cit.*), and the description of their morphological and other characters, is reserved for a future occasion.

Summary and Conclusion.

1. Evidence has been brought forward that in the enteric and dysenteric groups of bacteria dys-agglutinable and hyper-agglutinable forms or phases occur, and can be produced experimentally by the methods described.

2. Both forms may be obtained from one and the same eu-agglutinable strain of a bacillus, and both may revert, or may be converted mutually the one into the other.

3. In agglutination tests carried out in the ordinary manner, a highly dys-agglutinable bacillus may fail to agglutinate at all (at 1 in 25) with a serum that agglutinates the culture from which it was derived up to 1 in 1000 or more. It may also entirely fail to absorb from the serum any appreciable quantity of the agglutinins specific to that culture.

4. These results appear to necessitate a considerable modification of current theories regarding the value of absorption tests as a means of determining bacterial affinities, but may help to throw some light on the difficult problem of "serological strains." They show how necessary it is to reserve one's judgment, where conclusions are drawn regarding true differences of bacterial type, in cases where the differentiation rests solely on agglutination and absorption tests; since differences of such remarkable degree are shown to exist between different individuals among the population of a single culture.

I desire to express my indebtedness to Dr. A. D. Gardner, who kindly made independent readings of a number of my agglutination tests, and to Miss Edith F. Stubington for frequent and willing help in many of the more laborious experiments.

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The Titration Curve of Gelatine.—Report to the Medical Research Council.

By Dorothy Jordan Lloyd, Biochemical Laboratory, Cambridge, and Charles Mayes, Physical Laboratory, Eton College.

(Communicated by Prof. F. G. Hopkins, F.R.S. Received September 26, 1921.)

I. Introduction.

During the course of some work on the swelling of gelatine gels in acid and alkaline solutions, it became increasingly evident that no fully satisfactory theory of swelling could be postulated until further information had been obtained as to the quantitative relations, both general and particular, holding between the gelatine base and its combined acid in acid systems, and between the gelatine acid and its combined base in alkaline ones respectively. The work described in the following paper is an attempt to study the problem in its simplest form; namely, in a fluid system in which all the components are in solution. Hydrochloric acid and sodium hydroxide were chosen as the acid and base to use in the investigation, since both form highly ionised solutions in water, and since none of the ions resulting carry more than a single charge, thus simplifying any considerations deduced from the law of mass action. It is still an open question how far the law of mass action can be applied to colloidal solutions. It has been shown by Procter (20), Procter and Wilson (21), Wintgen and Kruger (27), that the quantitative relations found by them to exist in the combination of hydrochloric acid with gelatine, under the conditions of their experiments, fell within the general statement of the law, such combination being regarded as a simple case of salt formation.

Procter considered that his results were explicable on two hypotheses:—

(1) That gelatine had a molecular weight of 839, and combined with one molecule of hydrochloric acid to form gelatine hydrochloride; or

(2) That if gelatine had a larger molecular weight, some multiple of 839, say $839x$, then the gelatine molecule combined with x molecules of hydrochloric acid and the ionisation constants of the x hydroxyl groups involved must be the same. The second hypothesis he rejected as improbable.

We still consider that the molecular weight of gelatine must be greater than Procter has allowed, and we do not regard it as improbable that a number of the hydroxyl ions of the gelatine may have approximately equal ionisation constants. Our experimental results suggest that, up to a given concentration of hydrogen ions, a group of hydroxyl ions having approximately equal ionisation constants is involved; beyond this concentration, and up to a second fixed value, a second group approximating to a second constant is involved; and beyond this again there is slight evidence of a third group. The factors required in order to bring the second and possible third groups into conformity with the generalised statement of the law of mass action are not yet fully known.

II. Material and Method.

The gelatine used in this investigation was Coignet's Gold Label gelatine, purchased in 1914. It was purified by prolonged dialysis in dilute hydrochloric acid at a reaction of $C_H = 10^{-4 \cdot 6}$, and subsequent precipitation in strong alcohol. Details of the purification are given elsewhere (Jordan Lloyd, 7). It was dried with absolute alcohol and ether, and kept in a desiccator over pure sulphuric acid. (It is possible to cause the gelatine to lose more water by heating to 100° over phosphorus pentoxide *in vacuo.*) When dried with absolute alcohol it is a white brittle substance, fibrous in appearance, and containing 0·00 to 0·06 per cent. of ash. It forms clear solutions in water, which set to opaque white gels on cooling. The clear solutions set to colourless, glassy, transparent gels in the presence of free acid or base. The solution referred to below as 1 per cent. gelatine contains 1 grm. of this purified dry gelatine in 100 c.c. of freshly-boiled distilled water at room temperature.

The object of our experiments was to determine the amount of hydrochloric acid or sodium hydroxide which would combine with a constant weight of gelatine, and the method employed throughout was the electrical measurement of the concentration of the free hydrogen ions in solutions containing 1 per cent. of gelatine and known concentrations of hydrochloric acid or sodium hydroxide. The change of hydrogen ion concentration from that of an equally concentrated system containing no gelatine, is a measure of the acid (or base) which has combined with the gelatine. The routine method employed was to take 5 c.c. of a freshly made solution of 2 per cent. gelatine which had cooled but not yet set; the requisite amount of acid (or base) was added from a pipette calibrated to 0·01 c.c., and sufficient freshly-boiled distilled water to make the final volume 10 c.c. The reaction was taken at once by means of a gas chain. A Tinsley potentiometer was used.

The electrodes used were of a very simple modified Barendrecht type, and were made for us by Mr. A. W. Hall, of the Biochemical Laboratory. Their great advantage is the ease with which they are cleaned, a matter of great importance, as we found it essential to clean and re-coat the electrodes after every reading. A diagram of the electrodes is given below (fig. 1), and is self-explanatory.

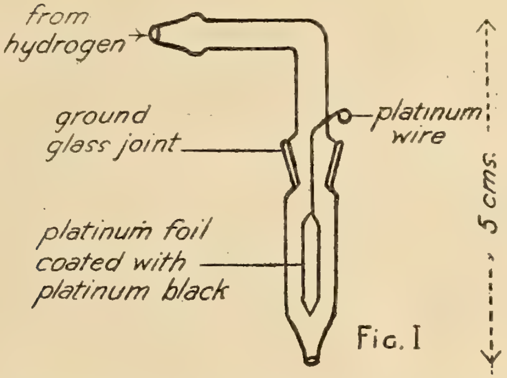


Fig. I

The platinum foil was coated very thinly with platinum black according to the directions of Michaelis (16). Only sufficient platinum was deposited to hide the glint of the foil. With such electrodes it was possible to take readings immediately contact had been made with the experimental fluid This is an essential condition for accuracy. With a slow electrode and a delayed reading, values tend to be very irregular. This is particularly marked in alkaline solutions. The experimental fluid was placed in silica cups into which the electrodes dipped. Contact was made by means of a sliding joint. The solutions had a strong tendency to froth near the iso-electric point. All experimental readings were taken at 20° C.

III. Experimental Results.

(a) *The Gelatine-Hydrochloric Acid Curve.*

If N represents the normality of a solution of hydrochloric acid and α its degree of ionisation, the concentration of the hydrogen ion present may be represented as $N\alpha$ on the normality scale, or $-\log N\alpha$ on the logarithmic scale. If 1 per cent. of gelatine be introduced into such a system, the reaction (P_H) is no longer given by the expression $-\log N\alpha$, but by some lower value. This can be determined by the hydrogen electrode.

The following experimental values were obtained for the variation of reaction (P_H) with total acid-content (N), gelatine concentration being kept constant at 1 per cent. temperature at 20° C. The constants used in calculating the reaction (P_H) from the observed electromotive force (E) are taken from Michaelis' 'Wasserstoffionen Konzentration' (16). The values for the concentration of the hydrogen ion are given on both the logarithmic scale P_H, and in terms of normality [H]. From the values of [H] it is possible to calculate the concentration of un-ionised free hydrochloric acid present in the system. If this is represented as [HCl], and α is the degree of ionisation of the acid, then

$$[\mathrm{HCl}] = [\mathrm{H}]/\alpha - [\mathrm{H}]. \tag{1}$$

The values of α are taken from Lewis's 'Text-book of Physical Chemistry' (3rd edition). Using Blasel and Matula's formula (1), if n' represents the concentration of acid removed from independent solution by the gelatine, then

$$n' = \mathrm{N} - [\mathrm{H}]/\alpha, \qquad \text{or} \qquad n' = \mathrm{N} - ([\mathrm{H}] + [\mathrm{HCl}]). \tag{2}$$

Equation (2) only holds if the value of α in (1) is the same both in the presence and absence of dissolved gelatine. In Table I, Column I gives the concentration of acid used, Column II the corresponding values of α, and Column III the values of $-\log \mathrm{N}\alpha$. Column IV gives the electrode reading in millivolts, and V the value of $\mathrm{P_H}$ calculated from the formula

$$\mathrm{P_H} = (\mathrm{E} - 248{\cdot}8)/58{\cdot}1.$$

Columns VI and VII give [H] and n' respectively.

The two curves $-\log \mathrm{N}\alpha : \mathrm{N}$ and $\mathrm{P_H} : \mathrm{N}$ from the values given in Table I are shown in fig. 2, and are designated as A and B respectively.

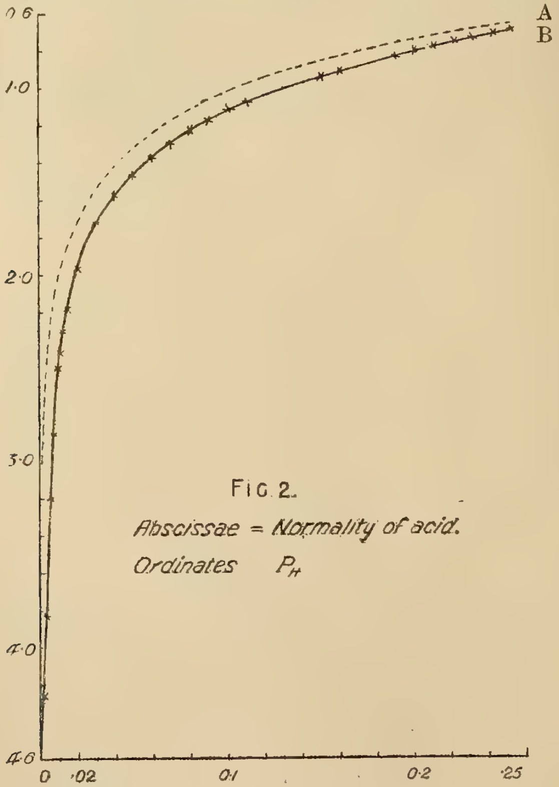
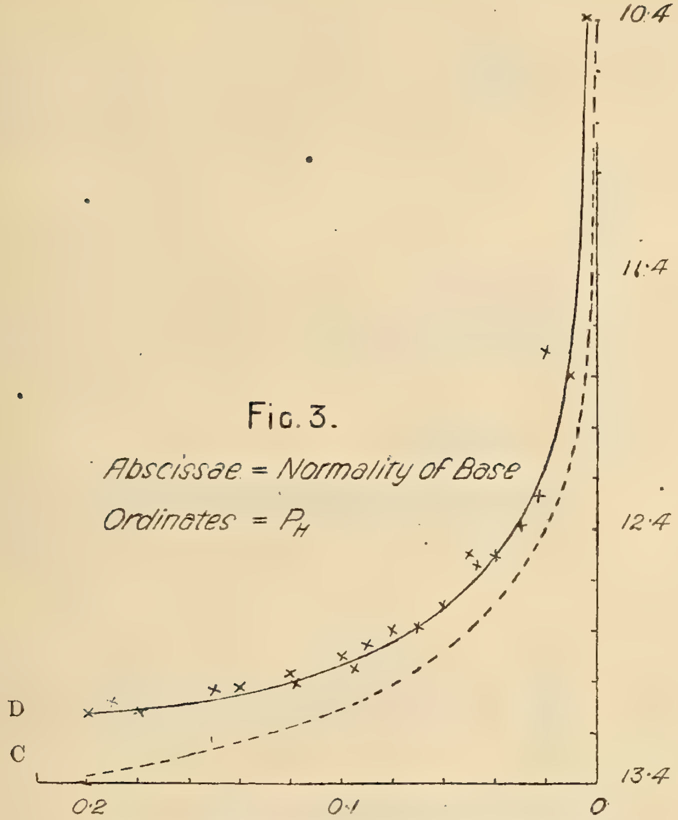


FIG. 2.

Abscissae = Normality of acid.

Ordinates P_H

Buffer curve of gelatine in hydrochloric acid.



Buffer curve of gelatine in sodium hydroxide.

Blasel and Matula, in calculating n', the hydrochloric acid fixed by gelatine, as $n' = N - [H]/\alpha$, assume that the value for α is the same when $[H] = [Cl]$ as when $[H] \neq [Cl]$, provided that the values for [H] are equal. This, however, is not true. A closer approximation is obtained by taking the square root of the product of [H] and [Cl] as the basis of the calculation. The value for [H] is obtained experimentally from hydrogen-electrode readings. The value for [Cl] is obtained by assuming that the gelatine chloride is completely ionised, and that therefore, $[Cl] = [H] + n'$. This assumption is also made by Procter and Wilson (21), though Bugarsky and Liebermann's experimental figures on the concentration of the chlorine ions do not support it (3). The amount of un-ionised acid [HCl] depends, therefore, not upon [H] but on $[H]_{corr.}$, where

$$[H]_{corr.} = \sqrt{([H] \times [Cl])},$$

and a new value for the acid fixed, say N′, follows as before. This is given in Column VIII and is shown plotted in fig. 4. [In the values for n' calculated

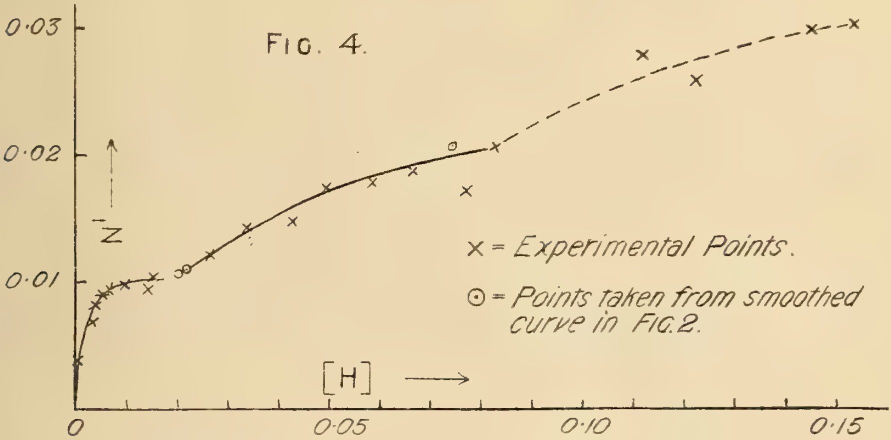
Table I.

I. Concentration of HCl = N.	II. Degree of ionisation = α.	III. $-\log N\alpha$.	IV. E.M.F. in millivolts of gas chain of 1 per cent. gel. solution = E.	V. $P_H = E-248{\cdot}8/58{\cdot}1$.	VI. [H.]	VII. $n' = N-[H]/\alpha$.	VIII. $N' = N-[H]_{corr.}/\alpha$.
0·0000		7·06	548·2	5·151			
0·0040	0·980	2·39	471·5	3·833	0·00015	0·00385	0·00383
0·0101	0·972	2·00	394·0	2·500	0·00316	0·00686	0·00679
0·0121	0·969	1·94	389·5	2·420	0·00380	0·00821	0·00812
0·0141	0·967	1·88	383·0	2·310	0·00490	0·00910	0·00898
0·0161	0·965	1·83	376·1	2·191	0·00644	0·00952	0·00939
0·0200	0·962	1·72	365·3	2·005	0·00988	0·00984	0·00970
0·02417	0·958	1·63	356·2	1·848	0·01419	0·00960	0·00946
0·0300	0·953	1·54	349·0	1·720	0·01514	0·0105	0·0104
0·0320				*1·69*	*0·0204*	*0·0108*	*0·0107*
0·0340				*1·66*	*0·0219*	*0·0112*	*0·0110*
0·0403	0·949	1·42	340·2	1·573	0·0267	0·0124	0·0120
0·0503	0·944	1·32	334·2	1·470	0·0339	0·0148	0·0142
0·0503				*1·455*	*0·0350*	*0·0135*	*0·0129*
0·0604	0·939	1·25	328·3	1·369	0·0428	0·0152	0·0147
0·0705	0·935	1·18	324·6	1·305	0·0496	0·0179	0·0173
0·0806	0·931	1·12	320·5	1·234	0·0583	0·0185	0·0177
0·0906	0·928	1·07	317·2	1·177	0·0665	0·0195	0·0186
0·1007	0·923	1·03	313·5	1·114	0·0769	0·0180	0·0171
0·1007				*1·13*	*0·0741*	*0·0215*	*0·0205*
0·1108	0·922	0·95	311·7	1·082	0·0828	0·0216	0·0206
0·1510	0·912	0·86	304·1	0·952	0·1117	0·0295	0·0278
0·1611	0·910	0·83	301·8	0·912	0·1225	0·0274	0·0258
0·1913	0·905	0·77	297·5	0·838	0·1452	0·0319	0·0298
0·2014	0·903	0·74	296·1	0·814	0·1535	0·0324	0·0302
0·2115	0·902	0·72	294·2	0·782	0·165	0·029	0·027
0·2215			292·4	0·750	0·178	0·024	0·022
0·2316			291·1	0·728	0·187	0·023	0·022
0·2417			290·1	0·711	0·194	0·027	0·025
0·2517			288·6	0·685	0·207	0·023	0·021

N.B.—Figures in italics are taken from the smoothed curve and not from experimental points.

from Blasel and Matula's formula it is assumed that the gelatine hydrochloride contributes no chlorine ions to the system; in those for N′ it is assumed that the gelatine hydrochloride contributes all its chlorine as free chlorine ions to the system. The two curves, n' : [H] and N′ : [H], therefore form the limits within which the actual curve must lie. In hydrochloric acid of concentrations less than 0·02 grm. of free hydrogen ion per litre the difference between the two limiting curves is negligible.] It can be seen that the curve is not a simple smooth curve, but that it consists of two, and possibly three, distinct regions. The deductions from this will be considered after the gelatine-sodium hydroxide curves have been described.

FIG. 4.



Curve of acid fixed. Abscissæ = [H]. Ordinates = Acid fixed or N′.

(b) *The Gelatine—Sodium Hydroxide System.*

Let N represent the normality of the caustic soda and α its degree of ionisation at 20° C., then $-\log N\alpha$ equals the hydroxyl ion concentration of the system and $14{\cdot}13-(-\log N\alpha)$ is the hydrogen ion concentration. Values for α are given by Kohlrausch (9), Noyes (18) and Jones (6). Unfortunately they differ considerably. The values given by Noyes were taken to plot the (broken) curve $14{\cdot}13+\log N\alpha$ in fig. 3 (marked C in the margin). Noyes' values only go to a concentration 0·05 N. The curve was extended beyond this region by taking Jones's figures for α and adding to them the difference between his figures and Noyes', which may be taken as 0·05 if α is expressed as fractions of unity. N, α and $14{\cdot}13+\log N\alpha$ are shown in the first three columns of Table II. The fourth column gives the readings obtained for E, the electro-motive force at the surface of the hydrogen electrode in a 1·00 per cent. solution of gelatine. The fifth column,

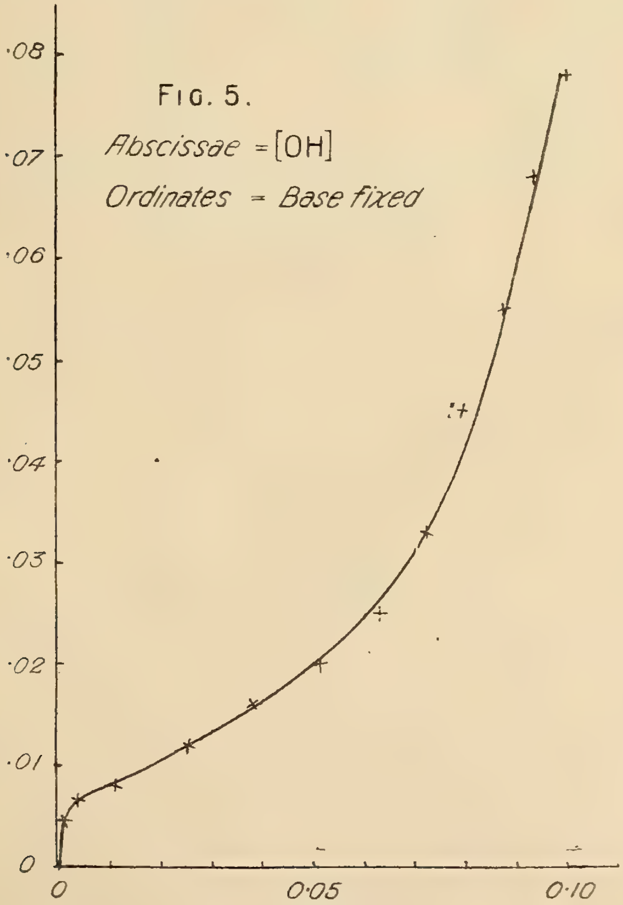
Table II.

NaOH = N.	Degree of ionisation = α.	14·13 + log Nα.	E.M.F. = E.	$P_H = E - 248·8/58·1$.		[OH] = antilog $-(14·13 - P_H)$.	NaOH unfixed = [OH]/α.	$n' = N - [OH]/\alpha$.
				Observed.	From smoothed curve.			
0·000			548·2	5·15				
0·002	0·984	11·42	557·2	5·30				
			685·0	7·50				
0·004	0·976	11·72	726·0	8·22				
0·005	0·974	11·82	852·0	10·39				
			969·0	12·40	*10·40*	*0·000*	0·000	*0·005*
0·006	0·972	11·90	786·0	9·24				
0·010	0·962	12·11	934·5	11·80				
			934·5	11·80	*11·65*	*0·003*	0·003	*0·007*
0·020	0·949	12·41	929·0	11·71	*12·17*	*0·111*	0·112	*0·008*
0·023	0·948		961·5	12·26				
0·030	0·939		969·0	12·39				
			952·0	12·06				
			975·5	12·50				
0·040	0·933	12·70	975·5	12·50				
			975·5	12·50	*12·53*	*0·025*	0·028	*0·012*
0·047	0·929		977·2	12·54				
0·050	0·923		975·5	12·50				
			975·5	12·50				
0·060	0·921	12·87	986·1	12·70	*12·71*	*0·038*	0·044	*0·016*
0·070	0·917		991·2	12·78				
			992·2	12·79				
0·080	0·913	12·99	993·5	12·80	*12·84*	*0·051*	0·060	*0·020*
0·090	0·910		995·8	12·85				
0·094	0·909		1001·1	12·95				
0·100	0·908	13·09	995·8	12·85				
			998·2	12·90	*12·93*	*0·063*	0·075	*0·025*
0·117	0·903		1002·5	12·97				
0·120	0·903				*12·99*	*0·073*	0·087	*0·033*
0·140	0·898		1005·6	13·02	*13·03*	*0·079*	0·095	*0·045*
0·150	0·896	13·26	1006·1	13·03				
0·160	0·893				*13·07*	*0·087*	0·105	*0·055*
0·180	0·890				*13·10*	*0·093*	0·112	*0·068*
0·190	0·888		1008·7	13·08				
0·200	0·887	13·37	1014·0	13·16				
			1011·4	13·12	*13·13*	*0·100*	0·122	*0·078*
0·400	0·84		1030·5	13·44		0·204	0·235	0·165
0·500	0·83		1036·8	13·54		0·257	0·300	0·200

N.B.—Figures in italics are taken from the smoothed curve and not from experimental points.

which is sub-divided, gives values for P_H calculated from E, and read from the smoothed curve N : P_H (marked D in fig. 3). The sixth column gives [OH] the concentration of hydroxyl ions on the normality scale, and the final column gives n', which is equated to $N-[OH]/\alpha$, and is the first calculation for the amount of base "fixed" by the gelatine.

In taking the "acid-fixed" values, the calculations in Table I were made directly from the experimental readings of E. These lie so very closely to a smooth curve that it is safe to assume that the experimental error is slight. In the case of the values of E in alkaline solution, the error is obviously very much greater. Chemical destruction of the gelatine by hydroxyl in the presence of spongy platinum is probably the cause, and hydrolysis has also been shown to have a slight effect on the reading. For this reason, an arbitrary smooth curve (P_H : N or D in fig. 3) was drawn through the



Curve of base fixed, n'.

experimental points, and the calculations of n' have been made from P_H readings taken from this smooth curve and not from the P_H readings given in Table II. Taking into consideration the poor quality of our experimental determinations of E in alkaline solutions, and the lack of agreement as to the values of α for caustic soda, it has not been considered worth while to correct the values of n' obtained by the Blasel and Matula formula. Fig. 5 shows n' plotted against [OH], and will be referred to later in Section IV (*b*).

IV. The Calculation of the Ionisation Constants of Gelatine.

(a) *The Value of* K_b.

The combination of gelatine with hydrochloric acid between 0·00 and 0·25 N concentration of acid may be represented as a curve with three sections.

Assuming for the moment that gelatine in hydrochloric acid solution behaves as a monacidic base from 0·00 to 0·04 N HCl, and combines with the acid to form an ionisable salt (see Introduction), the system may be represented as follows:—

Let [G(H)OH] represent the un-ionised gelatine base, and [GH′] and [OH′] the two ions of the ionised base; and assume that the salt GHCl is completely ionised; now by the law of mass action for a weak base,

$$\frac{[GH'] \times [OH']}{[G(H)OH]} = K_b,$$

$$\therefore \frac{[GH']}{[G(H)OH]} = \frac{[H]}{K_1} \text{ where } K_1 = \frac{K_w}{K_b}, \text{ the hydrolysis constant of the base.}$$

Let C = the equivalent concentration of the gelatine.

Then $[GH'] + [G(H)OH] = C$,

$$\therefore \qquad \frac{[GH']}{C} = \frac{H}{H+K_1}, \qquad (3)$$

or

$$\frac{[GH']}{[GH']+[G(H)OH]} = \frac{H}{H+K_1}.$$

Now, in equation (1), [GH′] is equal to N′ and is known, H is known, and therefore there are two unknown quantities, C and K_1. If Procter's (20) value of 839 be taken as the reacting weight of gelatine, C = 0·012. Wilson's (26) later value of 768 makes C equal to 0·013. Wintgen and Kruger (27), using the catalysis of methyl acetate as a measure of the concentration of the hydrogen ion, obtain a molecular weight of 839 for gelatine, while, calculating from the experimental results of Pauli and Hirschfeldt, they obtain the value 881·4. They give $2·7 \times 10^{-11}$ as a value for K_b at 25° C. In the calculations given below, C is taken as 0·0120.

Substituting for N′, H, G(H)OH in equation (1), we get the following values for K_1:—

N′.	[H.]	[G (H) OH.]	K_1.
0·00679	0·00316	0·00521	0·00242
0·00812	0·00380	0·00388	0·00184
0·00898	0·00490	0·00302	0·00165
0·00939	0·00644	0·00261	0·00180

K_1 may therefore be approximated to 0·0018,

whence $$K_b = \frac{K_w}{K_1} = \frac{10^{-14 \cdot 1}}{0 \cdot 0018} = 0 \cdot 48 \times 10^{-11}. \qquad (4)$$

A comparison of this value with Procter's value of $5 \cdot 2 \times 10^{-12}$ shows that they are both of the same order. It is a workable hypothesis to suppose that gelatine in the presence of hydrochloric acid, the concentration of which lies between 0·00 and 0·04 N, behaves according to the law of mass action, like a weak base with a reacting weight of 839 and ionisation constant of $4 \cdot 8 \times 10^{-12}$, each reacting mass combining with one equivalent of acid. Since we do not consider, on the chemical evidence at present available, that the molecular weight of gelatine can be less than 10,000, then it follows that, in its first stage of combination with hydrochloric acid, the gelatine molecule has available thirteen points of attachment for acid, all with a chemical potential very close to $0 \cdot 48 \times 10^{-11}$. Procter has considered this possibility, and rejects it as improbable, but, if we consider that the acid is attached to the free $-NH_2$ groups of the lysin and arginin, and possibly some other di-amino-acid, then it does not seem so improbable that the ionisation constants of these basic groupings might be of the same order. Kanitz (8) gives the following values for histidin, arginin and lysin :—

	First ionisation constant.	Second ionisation constant.
Histidin	$5 \cdot 7 \times 10^{-9}$	$5 \cdot 0 \times 10^{-13}$
Arginin	$1 \cdot 0 \times 10^{-7}$	$2 \cdot 2 \times 10^{-12}$
Lysin	$1 \cdot 0 \times 10^{-7}$	$1 \cdot 1 \times 10^{-12}$

These values are for the amino-acids in the free state. With arginin and lysin the first and second ionisation constants are of the same order of magnitude, hence it might be expected that even when bound by one amino-group into the protein molecule, the free amino-groups of both acids would have ionisation constants of the same order of magnitude. Evidence for the binding of the acid at these groups is given below.

The curve given by equation (3), *i.e.*, $\frac{[\mathrm{GH}']}{\mathrm{C}} = \frac{[\mathrm{H}]}{[\mathrm{H}]+0{\cdot}0018}$ gives a curve in which GH′ increases with [H] and only attains a maximum at infinite concentration of [H]. If we assume an error of 1 in 1000, [H] may be regarded as at infinite concentration when $[\mathrm{H}] = 0{\cdot}01$ N. The experimental curve, however, continues to rise very rapidly at still higher concentrations of acid. In his earlier papers Procter considers this difficulty and supposes that a second ionisation constant of a lower value also exists. But equations of the form $y = \frac{x}{x+a} + \frac{x}{x+b}$ where a and b are constants, give when plotted for x and y, a curve which rises rapidly at first and later more slowly, and it proved impossible to fit such a curve to the observations on fig. 4. These seem to indicate a maximum about $\mathrm{N}' = 0{\cdot}01$, then a gradual rise to a possible maximum about $\mathrm{N}' = 0{\cdot}02$ followed by yet another increase in N′. The observations for the latter part of the curve, however, are too uncertain to justify any definite conclusions.

(*b*) *Calculation of* K_a.

If J is the iso-electric point of an amphoteric electrolyte, K_a and K_b, its ionisation constants,

then
$$\mathrm{J} = \sqrt{\left(\frac{\mathrm{K}_a}{\mathrm{K}_b} \cdot \mathrm{K}_w\right)},$$

K_w at 20° C $= 0{\cdot}86 \times 10^{-14}$, K_b for gelatine at 20° C $= 4{\cdot}8 \times 10^{-12}$

$$\mathrm{J} = 10^{-4{\cdot}6}\ (17),$$

whence K_a should equal $3{\cdot}5 \times 10^{-7}$.

Now for a weak acid G (OH) H, we have

$$\frac{[\mathrm{H}'] \times [\mathrm{G\,(OH)}']}{[\mathrm{G\,(OH)\,H}]} = \mathrm{K}_a.$$

If [G(OH)′] is put equal to n', and C is put equal to [G(OH)′]+[G(OH)H], *i.e.*, to the initial concentration of the gelatine, then

$$\frac{n'}{\mathrm{C}-n'} = \frac{\mathrm{K}_a}{[\mathrm{H}]} = \frac{\mathrm{K}_a}{\mathrm{K}_w} \cdot [\mathrm{OH}].$$

But if 839 is taken as the reacting weight of gelatine, then $\mathrm{C} = 0{\cdot}012$, hence n' should be less than 0·012. But n' is already greater than 0·012 in 0·020 N sodium hydroxide and as N increases, n' increases, with an ever-increasing rapidity. Hence C cannot be taken as 0·012, but must be considerably greater. That is, the reacting weight in alkaline solution must be less than in acid solution, and hence different linkages must be involved.

If
$$\frac{n'}{C-n'}=\frac{K_a}{K_w}\,.\,[OH],$$
or $[OH]/K_2$, where $K_2 = K_w/K_a = 0{\cdot}86\times10^{-14}/3{\cdot}5\times10^{-7} = 2{\cdot}5\times10^{-8}$.

Then $C = (K_2+[OH])n'/[OH]$, and when we substitute the above value for K_2 we see that n' should be nearly equal to C at extremely small concentrations of hydroxyl ion and should then remain appreciably constant. This is not the case in fig. 5; here n appears to rise abruptly to about 0·005 and there seeks a maximum only to commence rising again to give a very steep curve.

Hence it is obvious that in alkaline solution gelatine does not behave simply as a weak acid dissociating in accordance with the law of mass action. It is possible that this abrupt rise accompanies some structural change of the protein molecule such as Dakin had shown to occur in strong alkaline solution (4). It must always be borne in mind that the hydrolysis of the —C(OH):N— groupings with the formation of free carboxylic and amino-groups occurs very rapidly in alkaline solution as measured by formaldehyde titration. Now the hydrolytic breakdown of the gelatine is not accompanied by a greatly increased basic binding power in the system, for a 1 per cent. solution of gelatine in sodium hydroxide having a reaction of $P_H = 12{\cdot}97$ was found after 3 hours at 100° C. to have changed to a reaction of 12·91. Further standing for 48 hours at room temperature was accompanied by a change of reaction to 12·90. This change in reaction corresponds to an increased combination of gelatine and base to the extent of only 0·011 equivalents of sodium hydroxide to 10 grms. of gelatine.

V. MECHANISM OF FIXATION OF THE HYDROCHLORIC ACID.

The most obvious points of attachment for acids in the gelatine molecule are the free amino-groups, and if hydrochloric acid forms salts with gelatine by addition of these groups, the salts should be regarded as hydrochlorides. Gelatine contains 18·0 per cent. of nitrogen in its total dry weight. According to Van Slyke and Birchard (25) 3·16 per cent. of this 18 per cent. (equal to half the lysin) can be removed as nitrogen gas by the action of nitrous acid, and can therefore be regarded as existing in the molecule in the form of free amino-groups. If these groups are the only ones in the molecule which can bind hydrochloric acid, then the maximum combining power of 10 grains of gelatine should be 0·0039 equivalents. Kossel and Cameron (11) and Kossel and Kellaway (12) have also brought forward evidence to show that proteins such as clupein and salmin, which contain no lysin, but which contain arginin, have a free amino-group in the guanidin nucleus of the arginin they contain.

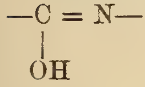
This group does not give off nitrogen gas under the action of nitrous acid (Van Slyke, 23). Kossel (10) states that the acid binding power of salmin is exactly that of its guanidin groups, and Kossel and Cameron (11) also consider that the free amino-groups of clupein must be those of its guanidin groups. Bracewell (2) considers that in all proteins the mechanism of acid fixation is by means of free amino-groups, and that in proteins such as gelatine, which contain both lysin and arginin, the acid-binding power should be given by the sum of half the lysin nitrogen plus one-quarter of the arginin nitrogen, each nitrogen atom binding one equivalent of acid. From this he calculates that since lysin contains 6·32 per cent. of the total nitrogen, and arginin 14·70, the maximum binding power of gelatine for acids should be 0·00085 equivalents per gramme, *i.e.*, 0·0085 equivalents for 10 grm. By a titrimetric method he finds that 1 grm. of undissolved gelatine powder can remove 0·00070 equivalents of acid from a supernatant solution.

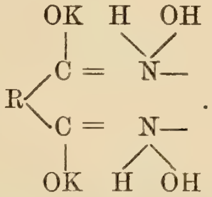
By the method which we have employed in this work the acid-binding power of gelatine, calculated on its lysin and arginin-content (which we find equal to 0·0086 equivalents for 10 grm. of gelatine), lies not at the true maximum of the $N' : [H]$ curve (fig. 4), but close to the first apparent maximum. It is possible, then, that in solutions of hydrochloric acid less than 0·02 N gelatine binds hydrochloric acid by means of its free amino-groups, and that it is the average ionisation constants of these basic groups that is given by the value $4{\cdot}8 \times 10^{-12}$. But with increasing concentration of acid, more acid is bound than can be accounted for on this hypothesis, and it is therefore necessary to consider what part the imino-nitrogen of the peptide linkage (—COHN—) could play. Robertson (22) states that the acid-binding power of proteins is not much increased by hydrolysis, and we have found that the reaction of a 1 per cent. solution of gelatine, which was found to be $P_H = 1{\cdot}13$, had only changed to $P_H = 1{\cdot}12$ after 7 hours at 100° C. This change is of the same order as the experimental error of the method, nevertheless hydrolysis of the gelatine had occurred during the heating in the strong acid solution, as was shown by the fact that the gelling power had been destroyed. It seems, therefore, that the peptide linkage can function as an acid-binding group.

Calculating again from Van Slyke's figures, if every amino-nitrogen atom of the free amino-acids of gelatine (*i.e.*, the amino-nitrogen from lysin and arginin + the imino-nitrogen from the peptide linkages) can act as a point for the fixation of an equivalent of acid, then 10 grm. of gelatine should be able to combine with 0·092 equivalents of hydrochloric acid. If only the di-amino acids (arginin and lysin, together with histidin) can do so, then the maximum value for acid fixed in a 1 per cent. solution of gelatine would be 0·020 equivalents. It can be seen from fig. 3 that the value 0·020 for N— corresponds to the maximum level of the second branch of the curve. The curve, however, continues to rise to a height of 0·030, though later it falls again. The experimental error in determining P_H becomes enormously magnified on the third limb of the [H] : N curve, and therefore too much stress cannot be laid on its smoothed form. However, it seems clear that some of the —COHN— groups can act as basic groups combining with acids. What *rôle,* if any, other groups (such as the hydroxyl groups of the hydroxy-acids) in the molecule play in acid fixation is still unknown. It will be necessary to follow experimentally the fate of the chlorine ion before final decisions are possible. At present our calculations of N′ involve the assumption that the gelatine hydrochlorides are present as completely ionised salts, an assumption that is liable to lead to an increasing error with increasing values of [H].

The Mechanism for the Fixation of Sodium Hydroxide.

The theory that proteins fix bases by means of their free carboxyl groups has given way on the accumulation of evidence that there are not enough of the latter to explain the quantitative relations

Brailsford Robertson (p. 236) suggests that the enolic imino-linkages  $\underset{\mathrm{OH}}{\underset{|}{-\mathrm{C}}}=\mathrm{N}-$, are responsible and " that the neutralisation of bases by the proteins is accomplished, at any rate for the greater part, by the dicarboxylic radicles which they contain." He gives the formula for potassium protein compounds as



According to the most recent analysis of gelatine (Dakin, 1920), the only di-carboxy acids present are aspartic acid (3·4 per cent.) and glutamic acid (5·8 per cent.). Calculating from these figures, the maximum combining capacity of a 1 per cent. solution of gelatine should be 0·0168 equivalents. On examining the curve of base fixed in fig. 4, it can be seen that this value for n' is by no means a maximum, but that instead the curve inflects and rises with increasing gradient. If every —COHN— group in the molecule is considered capable of acting as a point of attachment for bases, the maximum value for n' should be 0·09 ; n' however rises considerably above this figure. There must therefore be other means by which the gelatine molecule can fix sodium hydroxide. The possibility of linkage at some of the hydroxy-groups of the substituted amino-acids serine and hydroxyproline, is not to be ignored. Hydrolysis of gelatine by caustic soda has been shown to increase slightly its basic binding power, a fact which suggests that not all the —COHN— linkages are as potent as base fixers as the free —COOH— groups. Loeb (14, 15) has shown that bases react with gelatine at the same hydroxyl ion concentrations in equivalent proportions. This fact shows that the reaction is ionic, and that the compounds formed are of the nature of ionisable salts. Loeb only worked with solutions whose alkalinity was less than $P_H = 9$. His experimental values correspond very closely to our values over the same range.

The long slow rise of n' with increasing alkalinity is very striking. A feature in which the alkali-gelatine system differs markedly from the acid-gelatine system is illustrated by a different property of the gelatine, namely, the turbidity of the gel. Sols of 1 per cent. gelatine in distilled water set in a few hours to white, turbid gels. In the presence of 0·001 N hydrochloric acid the gel is not turbid, but quite clear and glassy after 24 hours' standing at 15° C. In the presence of caustic soda, however, the turbidity of the gel 24 hours old persists up to a concentration of 0·002 N soda. Thus there is both quantitative and qualitative evidence to show that in the same protein the mechanism of fixing acids is different from that of fixing bases.

VI. SUMMARY.

1. Hydrochloric acid combines with gelatine in solutions whose acid concentrations are less than 0·04 normal, according to the law of mass action. K_b for gelatine is $4{\cdot}8 \times 10^{-12}$ at 20° C. if 839 be taken as the reacting weight of gelatine.

2. The theory is put forward that over this range of the curve of combination of gelatine with hydrochloric acid, the combination occurs at the free $—NH_2$ groups. These groups are present in the lysin, arginin, and possibly some other of the amino-acids of the gelatine. The ionisation constants at these groups are taken as approximately equal. The salts formed are hydrochlorides.

3. In concentrations of hydrochloric acid greater than 0·04 normal, the proportion of acid fixed is greater than would follow from the combination of hydrochloric acid with a weak base, with an ionisation constant of $4{\cdot}8 \times 10^{-12}$. This is not due to the hydrolytic decomposition of the gelatine and release of further free $—NH_2$ groups. It seems possible therefore that combination is also occurring at the nitrogen of the peptide linkages.

4. In dilute sodium hydroxide of a concentration less than 0·01 normal, the gelatine combines with the base less rapidly than would follow by a

calculation from the value for K_b and the value for the iso-electric point, if 839 is taken as the reacting weight.

5. It is suggested that the number of positions of attachment for bases is not the same as the number of positions for acids, *i.e.*, that the reacting weight $= \dfrac{\text{molecular weight}}{\text{basicity (or acidity)}}$ is not the same in acid and alkaline solution.

6. It has not been found possible to calculate a value for K_a.

7. It would appear that in concentrations of sodium hydroxide about 0·1 N the structure of the molecule undergoes some change.

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The Hæmolytic Action of Sodium Glycocholate.

By Eric Ponder.

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(From the Department of Physiology, University of Edinburgh.)

Introduction.

This paper contains a detailed investigation into the action of sodium glycocholate, and into certain phenomena in which this salt plays an important part. The presentation of this research is a matter of some difficulty, since the observations recorded constitute merely the outlines of a very complex subject. It has been thought best, even at the expense of some lack of logical sequence, to present the problems more or less in the order in which they presented themselves for solution, the reader being thus taken over the several questions in the order in which they were investigated. To avoid undue length, no detailed description of methods used is given, if such description is to be found in a previous paper, on the findings of which this work is based (1).

The Physical Condition of Solutions of Bile Salts.

Although there is no definite statement on the subject, the general opinion appears to be that sodium taurocholate and sodium glycocholate form true solutions in water. If this be so, it is remarkable that they possess properties peculiar to colloids. If sodium taurocholate be dissolved in water, a clear yellow solution results. This clearness soon disappears, the solution becomes opalescent and, after about 12 hours, quite opaque. The opalescence is caused, presumably, by the taurocholate passing into a physical state other than that in which it was when first dissolved. The opalescent solution has all the properties of a taurocholate solution; its hæmolytic activity is as great as is that of a clear solution of the same strength. A difference appears on filtering the opalescent solution; the filtrate has a less hæmolytic power than the original solution. This fact, together with the opalescence, suggests that the taurocholate has assumed a less dispersed form.

The more dilute the taurocholate solution is made, the more rapidly does the opalescence appear; in solution in 1 per cent. saline a similar occurrence takes place, but less rapidly than in aqueous solution.

Sodium glycocholate behaves in a similar way, but the appearance of the opalescence is not so rapid. If in a 1 per cent. solution in saline, the opalescence takes days to appear.

Both salts possess a property peculiar to colloids—they protect a gold sol against precipitation by electrolytes. This protective power is shown in the following way. Using a gold sol prepared by the formaldehyde method, 1 c.c. is precipitated by 0·1 c.c. of 10 per cent. NaCl within 10 seconds. If small quantities of bile salts be added, we find that precipitation is prevented. This protective power is apparent before the solution becomes opalescent, and becomes less as opalescence proceeds, attaining a minimum after about 36 hours. The smallest quantity of taurocholate and glycocholate respectively which will protect 1 c.c. of gold sol against 0·1 c.c. 10 per cent. NaCl is shown in the following Tables, the solutions being kept at 18° C.:—

Table I.

Hours after preparation.	Smallest protecting quantity.	
	Taurocholate (1 in 1000).	Glycocholate (1 in 1000).
	c.c.	c.c.
1 hour	0·2	0·35
12 hours	0·3	0·4
24 ,,	0·35	0·55
36 ,,	0·4	0·6
48 ,,	0·4	0·6

It will be observed that (1) the taurocholate is about twice as protective as the glycocholate, and (2) that, as opalescence becomes established, there is a loss of protective power, the minimum being about half the protective power of the fresh solution.

A more complete study of the physical chemistry relating to this subject is being made.

The Hæmolytic Action of Sodium Glycocholate.

In a paper previously published (1), it has been pointed out that sodium glycocholate behaves in a manner similar to that found in the cases of sodium taurocholate, saponin, and other hæmolytic agents. In dilutions higher than 1 in 1000, the rapidity with which this salt produces hæmolysis depends on the dilution, there being a relatively simple relation between the two. When one examines the action of the salt in concentrations of 1 in 100, 1 in 500, etc., a different behaviour is observed: in three respects especially.

(1) There is great difficulty in obtaining consistent results; this difficulty does not ordinarily exist, for the time required for hæmolysis can, as a rule,

be observed correct to a few seconds, and readily reproduced with consistence. In the case of sodium glycocholate acting in these high concentrations, a variability in the time taken for hæmolysis appears, even when factors such as temperature are controlled.

(2) The salt hæmolyses more rapidly in dilutions of 1 in 1000 and thereabouts, than in dilutions of 1 in 100.

(3) A freshly prepared suspension of red cells seems to be less rapidly hæmolysed by the salt in all dilutions up to 1 in 1000, and especially in dilutions of 1 in 100, and 1 in 50, than is a suspension which is 12 or 18 hours old. This is not unexpected; the envelopes of these old cells being probably weak. The importance of using a freshly prepared suspension for quantitative hæmolytic tests was insisted on in a previous communication.

The following Table gives the times taken for complete hæmolysis of 0·2 c.c. of standard blood suspension, freshly prepared, by various dilutions of this salt, at 18° C.:—

Table II.

δ.	T.	δ.	T.
50	25 minutes.	400	40 minutes.
100	90 „	600	30 „
200	65 „	1000	10 „

It must be understood that this Table is merely representative of the general behaviour of the salt, and that the times given are not the same for all suspensions, but vary with the condition of the cells, etc.

These results may be looked upon as unexpected—hæmolysis occurring more rapidly with a dilution of 1 in 1000 than with a dilution of 1 in 100. It is obvious that the hæmolysis depends on some factor other than the simple solution of the lecithin and cholesterin envelope of the erythrocyte in the solution of bile salt (2). It will further be observed that sodium glycocholate is a feebly hæmolytic agent compared with sodium taurocholate.

It is with occurrences connected with the action of the glycocholate in these relatively high concentrations that this paper is concerned.

It is convenient here to insert a note regarding terminology. The letter T is used to denote the time required for the complete hæmolysis of the amount of blood suspension used. The letter τ represents the temperature at which the experiment is conducted. The symbol δ denotes the number of cubic centimetres which contain 1 grm. of a hæmolytic agent, in a solution which is being used to produce hæmolysis. For detail regarding this nomenclature, the reader is referred to a previous paper (1): in that paper the special technique employed in these hæmolytic experiments is given in full. In the case of sodium glycocholate, when working at temperatures in the neighbourhood of 20° C., the exact temperature is very important, as in this region a slight rise of temperature greatly accelerates the hæmolysis. Neglect of taking this into account is a fruitful source of error.

The Effect of Blood Serum on the Action of Bile Salts.

Blood serum exerts a powerful inhibitory influence on the hæmolytic action of sodium glycocholate and sodium taurocholate. This is illustrated by the following results, the experiment being carried out in the way indicated in a previous paper (1), and in this case at 18° C. :—

Table III.

	T.
Sodium taurocholate, 1 in 1000	3 minutes.
" " 0·1 c.c. serum	16 "
Sodium glycocholate, 1 in 1000	10 "
" " 0·1 c.c. serum	37 "

With a view to discovering which constituent of serum produced the inhibition, the inhibitory power, if any, of each constituent of serum was studied. This problem will be dealt with later: the subject for consideration at present being certain phenomena occurring when serum albumin is brought into contact with sodium glycocholate and a blood suspension. The fact that the bile salts cannot exert a hæmolytic action in the presence of blood serum is of great interest, since it throws new light on the controversies regarding hæmolysis and hæmoglobinuria in jaundice (3).

The Effect of Serum Albumin on the Hæmolytic Action of Sodium Glycocholate.

The following solutions are used :—

(1) Solutions of sodium glycocholate in saline (0·95 per cent. NaCl). The strength of these solutions is shown in the following Table :—

Table IV.

Glycocholate.	Final concentration.	Value of δ.
per cent.		
2·5	1 in 100	100
1·25	1 200	200
0·833	1 300	300
0·625	1 400	400
0·5	1 500	500
0·417	1 600	600
0·25	1 1000	1000

The second column gives the concentration of glycocholate in the tube if, of any of these dilutions, 2 c.c. be taken, and have 2 c.c. of saline, or saline solution of albumin, and also 1 c.c. of standard blood suspension, added. The third column gives the value of δ for such a mixture.

In all the experiments recorded below, for convenience, quantities one-fifth of these are used, the final concentrations being the same, *e.g.*, instead of 2 c.c. of glycocholate plus 2 c.c. of saline plus 1 c.c. of suspension, 0·4 c.c. of glycocholate, 0·4 c.c. of saline, and 0·2 c.c. of suspension are used.

(2) A solution of serum albumin in saline. The serum albumin was prepared from blood, dried, and kept for some months before use. The strength of the solution is 1 per cent.

(3) A standard blood suspension, as described in the previous paper (1). This is essentially a 5 per cent. suspension of thrice washed human erythrocytes in normal saline.

If to 0·4 c.c. of 2·5 per cent. glycocholate be added 0·2 c.c. of suspension, and, after an interval of 5 seconds, 0·4 c.c. of serum albumin solution be added, hæmolysis occurs very rapidly, in about 30 seconds. It has been noted above that a 1 per cent. solution of glycocholate takes over an hour to produce hæmolysis of this quantity of suspension. Since, after adding the albumin, the concentration of glycocholate is 1 per cent., and since hæmolysis occurs in about 30 seconds, it is obvious that the serum albumin solution has a powerful accelerating effect on the action of the bile salt. It may be observed that the serum albumin solution is of itself non-hæmolytic: and that the rapid hæmolysis is in no way explained by the fact that the blood cells remain in contact with a 2·5 per cent. solution of glycocholate for 5 seconds, since the salt in this concentration will not produce any hæmolysis in this short time. Control experiments, using saline instead of the serum albumin solution, render the accelerating action of the latter quite clear.

The occurrence of this rapid hæmolysis depends on several factors. The rapid hæmolysis occurs with a mixture of serum albumin, sodium glycocholate, and blood cells. These three substances may, however, be mixed in three different ways:—

Method 1.—Put 0·4 c.c. of glycocholate solution, 2·5 per cent., in a tube, add 0·2 c.c. of blood suspension, and then, after an interval add 0·4 c.c. of serum albumin solution.

Method 2.—Put 0·4 c.c. of serum albumin solution in the tube, add 0·2 c.c. of blood suspension, and after an interval, add 0·4 c.c. of 2·5 per cent. glycocholate solution.

Method 3.—To 0·4 c.c. glycocholate solution add 0·4 c.c. of the serum albumin solution, and then 0·2 c.c. of the blood suspension.

The interval is, for convenience, 5 seconds. In all three cases the composition of the final contents of the tubes is the same. Yet very different results appear.

By method 1, hæmolysis occurs in 30 seconds.

By method 2, hæmolysis occurs in 25 minutes.

By method 3, hæmolysis may occur in a short time as in method 1, or after a long time, as in method 2; the time is usually intermediate between the two.

It is thus obvious that two different phenomena are being observed, according as to whether the glycocholate or the albumin is first brought into contact with the cells. It is further obvious that method 3 is of no use for the giving of consistent results, as the time taken to produce hæmolysis varies under apparently the same set of circumstances. The results given by method (1) will first be considered, as being the more important.

The effects produced by varying the quantity of sodium glycocholate in the above experiment, may now be investigated.

In a series of tubes is placed 0·4 c.c. of varying dilutions of sodium glycocholate, as mentioned above; to each tube is added 0·2 c.c. of blood suspension, and after an interval of 5 seconds, 0·4 c.c. of 1 per cent. serum albumin solution is added. The observation of the 5 seconds interval is very important. The results are expressed in tabular form:—

Table V.

When $\tau = 18$.

δ.	T.	δ.	T.
100	1 minute.	400	55 seconds.
200	2½ minutes.	600	50 „
300	1½ „	1000	6 minutes.

It will be found that very rapid hæmolysis occurs with all the dilutions of the glycocholate used.

If the same suspension be tested in a similar way after it has stood for a few hours, a different state of affairs will be found; the blood cells have undergone a change on standing. This surprising result occurs with great regularity and, with patience, the stages of the change may be made out. As an example, below is given in tabular form, the behaviour of a freshly prepared standard blood suspension, as time elapsed. The time for hæmolysis of 0·2 c.c. of this suspension by 0·4 c.c. glycocholate of various concentrations plus 0·4 c.c. of 1 per cent. serum albumin, was estimated by method 1, as above, at intervals of 1 hour, 12 hours and 24 hours after preparation, as well as immediately after preparation. The results were as follows:—

Table VI.

When $\tau = 18$.

δ.	Time after preparation.			
	5 minutes.	1 hour.	12 hours.	24 hours.
100	1½ minutes	2½ minutes	18 minutes	40 minutes.
200	2 „	30 „	20 „	15 „
300	1 minute	4½ „	4 „	3 „
400	50 seconds	50 seconds	1 minute	1½ „
600	40 „	50 „	1 „	1½ „
1000	2 minutes	3 minutes	7 minutes	8 „

All blood suspensions exhibit this change, some in greater degree, some in less. The change is not one merely to be detected with care, but a very obvious one, which makes investigation into this subject very difficult, much experience being necessary to correctly interpret results. It will be seen from this Table—which gives a typical result—that the freshly prepared suspension is rapidly hæmolysed by all the concentrations of glycocholate, on the addition of the serum albumin, it may be therefore termed "sensitive." An old suspension, however, is not rapidly hæmolysed except by dilutions of glycocholate in the neighbourhood of 1 in 500; it may, therefore, be called, compared to the fresh suspension, an "insensitive" suspension. This meaning will be attached to these terms in the following pages.

At this point it will be convenient to deal with one essential difference between a sensitive and an insensitive suspension. When a standard blood suspension is prepared, blood is drawn into citrated saline, to prevent coagulation. The suspension is centrifuged, the cells washed thrice with saline, and the cells then added to normal saline (0·95 per cent. NaCl), to form a 5 per cent. suspension. This suspension is normally sensitive. If the blood be drawn into normal saline instead of into citrated saline, and the act of coagulation thus permitted, an insensitive suspension results after washing the cells, and preparing the suspension in the same way as before. This is a constant occurrence; the act of coagulation seems to determine that the suspension shall be insensitive. If the blood be drawn slowly from the finger and clotting thus be allowed to begin, the resulting suspension will be insensitive. This very interesting fact is of use; for, with reasonable care, a sensitive suspension can always be prepared, and also if an insensitive suspension be required, it can with certainty be made.

The changes through which a sensitive suspension goes on standing are very curious; further investigations, to be noted below, throw some light on these changes. It may be observed that there is no difference between a sensitive and an insensitive suspension as regards the activity of either sodium glycocholate or sodium taurocholate when acting upon it; the difference exists only towards the mixture of glycocholate and albumin. The above mentioned phenomena occur not only with human erythrocytes, but with the red cells of dogs, cats, rabbits and guinea-pigs. The length of time for which a sensitive suspension remains unchanged varies. Some suspensions become insensitive within half an hour of preparation; others remain unchanged for as long as 12 to 20 hours.

Having considered the effect of varying the amount of glycocholate used (Table V) when 0·4 c.c. of 1 per cent. albumin solution is used to accelerate the hæmolysis, it must now be considered what the effect is of varying the amount of serum albumin. The following Table shows this. The substances are mixed in the same way as that adopted for the drawing up of Table V, *i.e.*, by method 1; 0·4 c.c. of albumin is added in each case.

Table VII.

When $\tau = 18$.

Albumin.	Glycocholate.			
	1 in 100.	1 in 400.	1 in 600.	1 in 1000.
2 per cent.	30 seconds	20 seconds	35 seconds	5 minutes.
1 ,,	28 minutes	30 ,,	80 ,,	15 ,,
0·5 ,,	43 ,,	4 minutes	20 ,,	35 ,,
0·2 ,,	56 ,,	12 ,,	1½ minutes	17 ,,

From a consideration of this Table it is obvious that the phenomena are very complex. The subject will be left in the meantime, and referred to again later (Table XIV). It will be sufficient to note here that the quantity of serum albumin used in combination with various dilutions of glycocholate is of the greatest importance. In the above experiment the suspension used was an insensitive one.

Certain of the occurrences met with when the sodium glycocholate, blood cells, and albumin are mixed by method 2 may now be considered.

In this method, 0·4 c.c. of 1 per cent. solution of serum albumin is placed in a tube, 0·2 c.c. of standard suspension is added, and after five seconds, 0·4 c.c. of any desired concentration of sodium glycocholate is added. The results of various dilutions of glycocholate may be given; the suspension used is sensitive.

Table VIII.

When $\tau = 18$

δ.	Method 1.	Method 2.
	T.	T.
100	5 seconds	25 minutes.
200	10 ,,	30 seconds.
300	15 ,,	20 ,,
400	20 ,,	25 ,,
600	25 ,,	40 ,,
1000	$2\frac{1}{2}$ minutes	4 minutes.

The hæmolysis does not occur so rapidly with method 2 as with method 1. The difference is most marked when high concentrations of glycocholate are used. In investigating the sensitiveness of a suspension, therefore, it is very important that this difference be kept in mind.

The Protective Action of Serum Albumin.

It has been seen that while serum albumin, if added to a cell suspension in contact with sodium glycocholate, accelerates the hæmolysis produced by the latter, if it be added to a cell suspension it will protect it against the action of the sodium glycocholate and serum albumin mixture.

To investigate this further, one may put up four tubes, as follows:—

Tube 1.—1 c.c. suspension 0·2 c.c. of 1 per cent. serum albumin.

Tube 2.—1 c.c. suspension 0·1 c.c. of 1 per cent. serum albumin.

Tube 3.—1 c.c. suspension 0·05 c.c. of 1 per cent. serum albumin.

Tube 4.—1 c.c. suspension 0·02 c.c. of 1 per cent. serum albumin.

The suspension used is a sensitive one. Allow all tubes to stand for 5 minutes. Examine the suspensions to see if they are sensitive or insensitive. The following result is typical:—

Table IX.

When $\tau = 18$; $\delta = 100$; $+0{\cdot}4$ c.c. 1 per cent. albumin.

Tube.	T.	Tube.	T.
Control	15 seconds.	3	5 minutes.
1	40 minutes.	4	1 minute.
2	22 ,,		

The effect, then, of adding a small quantity of serum albumin solution to a sensitive suspension is to render it insensitive. The degree of insensitiveness produced depends on two factors: (1) the amount of serum albumin added; and (2) the time for which it remains in contact with the cells. A suspension thus made insensitive is not resensitised by washing once in the centrifuge: repeated washings may render it sensitive to some degree.

The Protective Power of Blood Serum.

As serum blood albumin has this effect, it might be supposed that blood serum itself had a similar effect. The following typical experiment shows this clearly:—

Tube 1.—1 c.c. suspension 0·02 c.c. serum.
Tube 2.—1 c.c. suspension 0·015 c.c. serum.
Tube 3.—1 c.c. suspension 0·01 c.c. serum.
Tube 4.—1 c.c. suspension 0·005 c.c. serum.

Allow the tubes to stand 5 minutes. The suspension used is a sensitive one. Test the suspensions to see if they are sensitive or not.

The typical result is as follows:—

Table X.

When $\tau = 18$; $\delta = 100$; $+0·4$ c.c. 1 per cent. albumin.

Tube.	T.	Tube.	T.
Control	15 seconds.	3	6 minutes.
1	20 minutes.	4	25 „
2	15 „		

The serum thus renders a sensitive suspension insensitive. Such an insensitive suspension is however restored on washing the cells in the centrifuge; an occurrence which is not found in the protection conferred by serum albumin, and suggesting that the protection is conferred in different ways in the two cases. The protection is conferred not by fresh serum only, but by preserved serum, kept for over 18 months by the method of Leers (4).

Animal Experiments.

It is confirmatory of these experiments that the protective action of serum albumin occurs *in vivo* as well as *in vitro.*

If a rabbit be taken and from a vein a small quantity of blood (0·05 c.c.) be drawn into citrated saline, the suspension resulting after washing these cells—preferably once—is sensitive to the mixture of glycocholate and serum albumin. If now 5 c.c. of a 2 per cent. serum albumin solution in saline be injected into a vein, and blood withdrawn from a distant vein about 5 minutes later, the suspension prepared from this blood will be insensitive.

Results for four rabbits may be recorded:—

Table XI.

When $\tau = 18$.

Rabbit.	T, 1st sample.	T, 2nd sample.	Albumin injected.
1	15 seconds	24 minutes	0·1 gramme.
2	20 ,,	15 ,,	0·04 ,,
3	15 ,,	23 ,,	0·08 ,,
4	30 ,,	18 ,,	0·04 ,,

The injection seems to cause no untoward effect on the rabbit, which is under chloroform anæsthesia.

These phenomena will be found to be not peculiar to serum albumin: other similar substances act as powerful accelerators of glycocholate hæmolysis and as protective agents, when used differently, just like serum albumin. Peptone is such a substance: its actions are exactly parallel with those of the albumin. On trying the effects of various animal extracts, similar properties were found to be possessed by both adrenalin and pituitrin (Parke, Davis preparations). Since it has been shown that pituitrin contains histamine, this at once suggests the possibility of the phenomena being due to histamine or histidine, appearing in both the albumin, the peptone, and the pituitrin (5).

The Effect of Histamine on Glycocholate Hæmolysis.

A series of solutions of histamine (Burroughs Wellcome) is prepared, the following dilutions being convenient:—1 in 500, 1 in 1000, 1 in 2000, 1 in 5000, 1 in 8000, and 1 in 10,000. These solutions are made in normal saline (0·95 per cent. sodium chloride).

Histamine and histidine are, *per se*, non-hæmolytic. If 0·4 c.c. of 2·5 per cent. glycocholate solution have 0·2 c.c. of standard blood suspension added, and if, after 5 seconds, 0·4 c.c. of 1 in 1000 histamine be added, hæmolysis occurs instantaneously.

The histamine thus has the accelerating action found with the serum albumin, whose action was probably due to the histamine contained in it as an unavoidable impurity. This is a most interesting fact, for it enables many more exact observations to be made on the phenomena mentioned above in connection with serum albumin. Certain preliminary notes must first be made.

The Effect of Histamine on Colloid Gold.

In view of the fact that sodium glycocholate protects a gold sol, it is important to know the action of histamine on such a colloid. Histamine precipitates colloid gold, acting powerfully in this respect. Histidine acts in a similar way, but less powerfully. The precipitating action of a 1 in 1000 solution of histamine is very marked, 0·1 c.c. precipitating 1 c.c. of the gold sol used in about 10 seconds.

It is probable, therefore, that histamine acts as a disturber of all colloids, and therefore of sodium glycocholate. We have seen that the hæmolytic action of this substance when in "combination" with serum albumin or histamine is not to be accounted for by simple solubility of the envelope of the erythrocyte in the bile salt: it is possible that surface tension produces the effect, in which case the interaction of a colloid like the glycocholate, and a precipitator of colloids such as histamine, would be of great interest. The consideration that the hæmolysis may be due to changes in the physical state of the solution, connected with occurrences known to colloid chemistry, suggests that the acidity or alkalinity of the hæmolysing solution will be of great importance: the phenomena perhaps being analogous to those of adsorbtion (6).

It will therefore be necessary to investigate (1) the action of serum albumin, and of histamine, on a blood suspension subjected to the hæmolytic action of various amounts of sodium glycocholate; and (2) the effect of acidity or alkalinity on this action.

The following Tables contain such an investigation. The suspension used is an inactive one. The various concentrations of glycocholate are similar to those previously used (Table IV). The quantity of serum albumin added to each tube, in the columns relating to its action, is 0·4 c.c. of a 1 per cent. solution in saline. The quantity of histamine added to each tube, in the columns relating to its action, is 0·4 c.c. of a 1 in 5000 solution in saline.

"Acid histamine," or "acid serum albumin," is made by adding to 10 c.c. of the histamine solution, 1 in 5000, or, to 10 c.c. of the 1 per cent. albumin solution, 0·1 c.c. of decinormal HCl. "Alkaline histamine," or alkaline serum albumin, is prepared by adding to 10 c.c. of either substance in the concentrations mentioned above, 0·1 c.c. of 1 per cent. Na_2CO_3.

The substances were mixed in the order referred to as method 1, *i.e.*, the glycocholate first, then the blood suspension, of which 0·2 c.c. is used, and then, after 5 seconds, the serum albumin or histamine.

Table XII.

When $\tau = 18$.

δ.	Albumin.	Histamine.	Acid albumin.	Alkaline albumin.	Acid histamine.	Alkaline histamine.
100	65 mins.	95 mins.	43 mins.	105 mins.	55 mins.	110 mins.
200	40 ,,	30 ,,	3 ,,	76 ,,	40 secs.	73 ,,
300	9 ,,	90 secs.	30 secs.	58 ,,	30 ,,	50 ,,
400	1 min.	40 ,,	20 ,,	35 ,,	15 ,,	15 ,,
500	50 secs.	30 ,,	30 ,,	18 ,,	30 ,,	4 ,,
600	1 min.	35 ,,	50 ,,	10 ,,	50 ,,	45 secs.
1000	5 mins.	3 mins.	3½ mins.	23 ,,	2½ mins.	1½ mins.

This Table, which is representative of the general results obtained with an inactive suspension, expresses several important points:—

(1) Histamine behaves similarly to the serum albumin, as an accelerator of the glycocholate hæmolysis.

(2) The rendering of the hæmolysing solution acid causes hæmolysis to be more rapid: if the hæmolysing solution be alkaline, the hæmolysis is retarded. The amount of deviation from neutrality is very small in the above case.

(3) The relation between the speed of hæmolysis under the various conditions and the amount of glycocholate used is expressed. Columns 1 and 2 are confirmatory of the Tables illustrating the behaviour of an inactive suspension.

The question of the reaction of the hæmolysing fluid is obviously one of great importance. A series of observations in which the P_H is determined would be ideal: the difficulties attendant upon the use of buffer solutions in connection with these hæmolytic experiments are at present, however, insuperable. The question is being investigated.

It now remains to consider the effect of varying the quantities of histamine employed for accelerating the glycocholate in its hæmolytic action. This is done in the following two Tables, the first Table illustrating the results when a sensitive suspension is used, the second illustrating the results in the case of an insensitive suspension. In each case, the temperature at which the experiments were conducted was 18° C.: the substances were mixed by method 1.

Table XIII.—A. Sensitive Suspension.

Histamine.	Glycocholate.						
1 in	1 in 100.	1 in 200.	1 in 300.	1 in 400.	1 in 500.	1 in 600.	1 in 1000.
500	5 secs.	5 secs.	3 secs.	8 secs.	10 secs.	25 secs.	40 secs.
1,000	7 ,,	7 ,,	5 ,,	15 ,,	20 ,,	35 ,,	1½ mins.
2,000	8 ,,	10 ,,	10 ,,	20 ,,	25 ,,	30 ,,	2 ,,
5,000	45 mins.	15 mins.	30 ,,	30 ,,	28 ,,	20 ,,	1½ ,,
8,000	50 ,,	18 ,,	45 ,,	45 ,,	30 ,,	25 ,,	1 min.
10,000	56 ,,	24 ,,	2½ mins.	2 mins.	1 min.	30 ,,	50 secs.

Table XIV.—B. Insensitive Suspension.

Histamine.	Glycocholate.						
1 in	1 in 100.	1 in 200.	1 in 300.	1 in 400.	1 in 500.	1 in 600.	1 in 1000.
500	5 secs.	5 secs.	5 secs.	8 secs.	10 secs.	15 secs.	40 secs.
1,000	7 ,,	7 ,,	10 ,,	15 ,,	18 ,,	28 ,,	1½ mins.
2,000	8 ,,	10 ,,	15 ,,	20 ,,	25 ,,	30 ,,	2 ,,
5,000	85 mins.	40 mins.	1 min.	30 ,,	25 ,,	23 ,,	1½ ,,
8,000	90 ,,	42 ,,	5 mins.	45 ,,	30 ,,	25 ,,	1 min.
10,000	105 ,,	45 ,,	7 ,,	2 mins.	1 min.	30 ,,	50 secs.

From these somewhat complicated Tables very little new is to be learnt, except that the occurrences which take place when the action of glycocholate of sodium is accelerated by histamine are exceedingly complex. Several points may, however, be noted:—

(1) If these times be plotted on graph paper, a series of curves of definite character will be obtained. The character of the curves, however, does not suggest any generalisation.

(2) The difference between the sensitive and the insensitive suspension may be seen in columns 1, 2 and 3 of the respective Tables. At the other dilutions these differences diminish. This is confirmatory of the observations made with serum albumin.

(3) From an inspection of columns 1 and 2, it appears that there is a very great disproportion between the effect produced by a 1 in 2000 histamine solution and a 1 in 5000 solution. This suggests that the occurrences met with when 1 in 500 to 1 in 2000 histamine is used are different in kind from those met with when more dilute histamine solutions are used, whereas the difference between the activity of the solutions to which the other columns relate is one of degree only. This is very probable, in the light of other considerations, and will be commented on later.

The Protective Action of Histamine.

It has been shown that histamine possesses the accelerating action on the hæmolysis produced by sodium glycocholate, being in this respect similar to the action of the serum albumin solution dealt with in the beginning of the paper. It remains to be shown whether or not it has the protective action of the serum albumin solution (Table IX).

This is a simple matter, in view of the information conveyed in Table XIII. The following experiment illustrates this:—

To 1 c.c. of an active standard blood suspension, add 0·1 c.c. of 1 in 500 histamine. Allow this tube to stand for 5 minutes.

If 0·2 c.c. of this suspension be added to glycocholate and histamine in a tube, it will carry with it a small amount of additional histamine. For instance, if 0·4 c.c. of glycocholate have added 0·2 c.c. of this suspension, and also 0·4 c.c. of 1 in 5000 histamine, the final dilution of histamine in the tube will be 1 in 8330, instead of 1 in 12,500, which it would have been if instead of this suspension containing histamine, a standard suspension had been used. Consulting Table XIII, it will be seen that if 1 in 500 glycocholate be employed for hæmolysis, this slight increase in the histamine concentration is of little consequence, altering the hæmolytic time only by 1 or 2 seconds. Therefore the effect of a dilution of 1 in 500 glycocholate on the standard suspension, before and after it has had histamine added in this quantity, will decide whether or not the histamine has caused a protection of the blood cells.

To two tubes, then, add 0·4 c.c. of a 0·5 per cent. solution of sodium glycocholate; add in the case of one tube, 0·2 c.c. of standard suspension, and in the case of the second, 0·2 c.c. of the suspension containing histamine, as prepared above; after a 5 seconds' interval, add 0·4 c.c. of 1 in 5000 histamine. The tube containing the standard suspension hæmolyses in 30 seconds, that containing the suspension, plus histamine, in 3½ minutes. This demonstrates that the histamine has a protecting effect on the cells against hæmolysis by the glycocholate-histamine system.

If the cells of this suspension plus histamine be washed in saline in the centrifuge, the suspension resulting from adding them to the appropriate amount of saline is still slow in being hæmolysed by these quantities of glycocholate and histamine. This demonstrates that, as in the case of the protection conferred by serum albumin solution, the protection conferred by the histamine is not due merely to the presence of the latter in the saline surrounding the red cells, but due to some change produced in the cells themselves.

The Action of Histidine.

Histidine acts in a manner similar to histamine in accelerating hæmolysis by sodium glycocholate, and, if used in another way, in protecting cells against hæmolysis by histidine and glycocholate. In general, its action in these respects is less marked than that of histamine.

These properties belonging to histamine and to histidine do not appear to be general properties of amino-acids. Glycine and arginin, for instance, do not possess them. A full study of this question is being made, and the point will not be further dealt with in this paper.

Discussion.

It is a much easier matter to observe the phenomena described in this paper than to explain them. A brief discussion of certain points is desirable.

It appears obvious that the explanation of hæmolysis by sodium glycocholate on the grounds that this salt dissolves the envelope of the corpuscle is inadequate, in view of the unusual behaviour of the salt in certain high concentrations, and especially in view of the action of non-hæmolytic substances like histamine when in the presence of a blood suspension and sodium glycocholate. A more probable explanation is one which is based on changes of surface tension; possibly the solvent action of the salt plays a subsequent part. To advance a theory to explain these occurrences is at present impossible; the following suggestion, however, is supported by the majority of the facts, and may be taken as a working hypothesis, useful for the present until further facts are brought to light.

If we consider first the addition of blood cells to a solution of sodium glycocholate, we may say that two occurrences take place: (1) the glycocholate becomes condensed at the interfaces, and therefore on the surface of the red cells; and (2) a solvent action of the glycocholate on the envelope of the cell begins. If histamine, which it has been seen, powerfully disturbs colloid solutions, be added, the colloidal glycocholate probably undergoes a sudden change of physical state, resulting in a sudden variation of the surface tension at the red-cell interfaces, where the glycocholate is collected. This sudden alteration ruptures the cell wall, all the more so as the glycocholate is already attacking the envelope, and therefore is, as it were, continuous with the substances composing it. The rapid hæmolysis produced by the addition of histamine may be thus explained. If, on the other hand, the histamine be added to the cells first, it will not be so condensed at the interfaces as the colloid would be, and will certainly not dissolve the envelope. On the addition of glycocholate, then, a change of physical state of the latter occurs, with a sudden change of surface tension, as before, but more evenly distributed throughout the fluid, instead of being more marked at the cell interfaces. Hæmolysis will therefore be slower; the possibility that the glycocholate and the histamine may form some species of adsorption compound which has scarcely any hæmolytic action might further enter into the explanation.

Such a consideration is further supported by the fact that the occurrences are so influenced by small changes in reaction; the process of adsorption and similar colloid phenomena being very sensitive in this respect. It may be also noted that the curious results obtained by varying the quantities of the interacting substances point to changes more complex than simple chemical interaction. It appears at times that the phenomena do not occur until certain quantities of the interacting substances are present, *e.g.*, in Tables XIII and XIV, columns 1 and 2. Here possibly the amount of histamine added was insufficient to disturb the glycocholate sufficiently to cause the change of surface tension necessary to produce hæmolysis.

Not the least interesting of these occurrences is the change which seems to occur in the blood cells themselves, both on standing and under the action of histamine. The latter occurrence seems to have no explanation which is even probable at present. The fact that blood cells, prepared in such a way that coagulation is permitted, are insensitive, may be due to some protective action exerted by some product of coagulation.

The whole subject, which may at first sight seem of little practical consequence, is of great importance. The facts show that hæmolysis by simple chemical substances depends on complex factors, and any information which can be gained regarding the true manner of action in these relatively simple cases is of interest in the consideration of the vastly more complex phenomena of hæmolysis by hæmolysins of animal origin.

Summary.

1. Sodium taurocholate and sodium glycocholate are to be considered as colloids. They protect colloid gold against precipitation of electrolytes.

2. Sodium glycocholate is a feebly hæmolytic agent. If histamine or histidine be added to it in suitable proportions, a highly hæmolytic mixture results, although histamine and histidine are of themselves non-hæmolytic. The reaction of the hæmolysing fluid influences the speed of hæmolysis.

3. Histamine, if brought in contact with blood cells, renders them immune to hæmolysis by the histamine glycocholate mixture. Histidine acts similarly. This appears to be due to some change in the cells themselves, and not merely to the presence of the histamine in the fluid.

4. Blood cells which are rapidly hæmolysed by glycocholate and histamine become insensitive on standing, but to a less degree with an old suspension than with a freshly prepared one.

5. Serum albumin and peptone solutions, and also pituitrin, produce both the rapid hæmolysis when mixed with sodium glycocholate, and also the protective effect when added to blood cells. This latter occurs *in vivo* as well as *in vitro.* These occurrences may be due to the presence of histamine or allied substances.

6. Several facts suggest that these phenomena are mainly due to disturbance of surface tension, similar to those which are met with in colloidal solutions. They cannot be explained by the theory that the bile salt dissolves the corpuscle envelope.

7. Suspensions of cells which are derived from blood drawn into a fluid which prevents coagulation behave differently from suspensions of cells which are derived from blood drawn into a fluid which permits coagulation to occur.

8. A protection against hæmolysis by the histamine-glycocholate mixture is also conferred by blood serum.

9. The presence of blood serum inhibits the hæmolytic action of sodium taurocholate and sodium glycocholate.

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The Mechanism of Ciliary Movement.

By J. Gray, M.A., Fellow of King's College, Cambridge, and Balfour Student, Cambridge University.

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The mechanism of ciliary movement has been extensively studied from the morphological point of view, and although there is a general consensus of opinion as to the structure of the "ciliary apparatus," there is no adequate account of the functions of the various parts of the mechanism.

The material used for this work has been the gills of *Mytilus edulis*, and has already been described (Orton, 27). It is entirely due to the movement of the cilia that an efficient stream of water is kept passing on to the face of the gill, and that the food is moved up to the mouth of the animal. By means of carmine particles the existence of these currents is easily detected by the naked eye.

The production of a constant current of water in a definite direction implies that the cilia are capable of performing work in a remarkably efficient manner. If we watch the movement of a single cilium, it is obvious that the beat is divisible into two phases: (*a*) a very rapid forward or effective stroke; and (*b*) a slower backward or recovery stroke. It is during the rapid effective stroke that the cilium performs work on the surrounding medium, and in doing so, of course, expends energy. At the conclusion of the effective stroke these cilia possess no energy which can be used for work, but by the time the recovery stroke is completed a new supply of potential energy is available and is in turn converted into kinetic energy during the next effective stroke.

We are, no doubt, entitled to assume that the energy expended by a cilium has its origin in some chemical compound, either in the cilium itself or in the cell to which it is attached. Our main problem is to throw what light we can on the sequence of events which leads to the conversion of chemical energy into the kinetic energy of movement.

The first evidence which will be presented is that gained by an observation of the living cells under normal conditions.

I. The Structure and Behaviour of Normal Cilia.

On the gill filaments are three main groups of ciliated cells—the lateral, the latero-frontal, and the frontal cilia (see Gray, 12). These cilia, like all other living cilia, appear to be optically homogeneous; they are strongly refractive and possess a considerable degree of elasticity. In this respect Engelmann (8) expressed the view that: "Tous les organes vibratils sont résistants, très flexibles, et dans une large mesure parfaitement élastiques." The cilia of *Mytilus* are entirely independent of any control by the animal and are in constant motion during the life of the cells.

Lateral Cilia.

On the sides of each gill filament are three rows of rectangular cells, each bearing a brush of cilia. These are the lateral cilia (see figs. 1 and 2). The effective stroke causes a strong current of water to flow on to the gill surface at right angles to it (see fig. 2). All the cilia arising from a single cell beat in the same phase, as do also the cilia of the three cells comprising each vertical row.

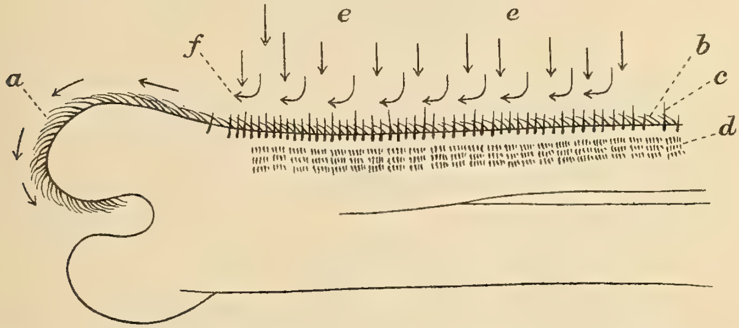

Fig. 1.—Lateral view of gill filament of *Mytilus* (modified from Orton). (*a*) Terminal cilia, (*b*) frontal cilia, (*c*) latero-frontal cilia, (*d*) lateral cilia, (*e*) vertical water current set up by lateral cilia, (*f*) direction of water current from frontal cilia.

The most distinctive feature of the lateral cilia is their marked rhythm. The cilia on adjacent cells beat in succession, so that a continuous wave passes along the whole line of lateral cells from one end of the filament to the other; the wave passes in opposite directions on the two sides of the filament. This metachronial rhythm provides an interesting example of co-ordinated movement which is not associated with any visible nervous elements. Isolated individual cells from the lateral epithelium continue to exhibit active movement.

Latero-frontal Cilia.

It will be seen from fig. 2 that on reaching the surface of the gill the water columns set in motion by the lateral cilia meet the large *latero-frontal cilia.* During one phase of the beat these cilia rapidly pass from the form of straight rods to that of curved hooks, the point being directed towards the free surface of the filament. The cilia then flatten out more slowly; the flattening begins at the base and proceeds to the point. These cilia also exhibit a certain degree of metachronism, but to a much less marked extent than the lateral cilia. The function of the latero-frontal cilia appears to be two-fold: (i) they act as vanes which deflect the water currents on to the surface of the filaments; (2) they keep the individual filaments apart, so as to give free play to the lateral cilia.

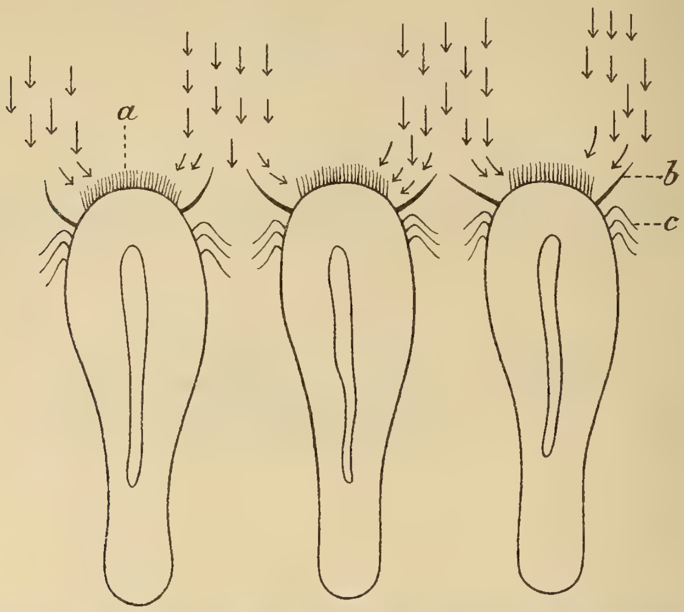

Fig. 2.—Transverse vertical section of three gill filaments, showing the deflection of the vertical current on to the frontal cilia. (*a*) Frontal cilia, (*b*) latero-frontal cilia, (*c*) lateral cilia.

Frontal Cilia.

The whole of the flat frontal surface of the gill is covered by the frontal cilia, whose effective beat is parallel to this surface and directed towards its free edge (see fig. 1).*

When the movement is very greatly reduced in speed by the addition of gum arabic to sea-water, it is seen that, during the effective beat, the cilium behaves as a more or less rigid rod, which moves forward on a pivot at its base. During the recovery stroke, however, the cilium assumes entirely different properties—it is drawn back as a limp non-elastic body in which a stress is set up which starts at its base and is transmitted to the free end, exactly as is the case in a fishing line during the backward movement of a cast. As the cilium moves back it loses its limpness, and at the end of the recovery stroke possesses a considerable degree of rigidity (fig. 3).

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* At the end of the filaments the frontal cilia are modified so as to deflect the current towards the food grove and towards the oral end of the gill. These modified frontal cilia are very obvious, and will be referred to as the terminal cilia.

When movement is taking place fairly quickly the cilium does not appear to straighten out completely at the end of the recovery stroke, but moves forward in the form of a sickle. During the final phase of the effective beat the hooked shape is always lost and re-develops during the recovery stroke.

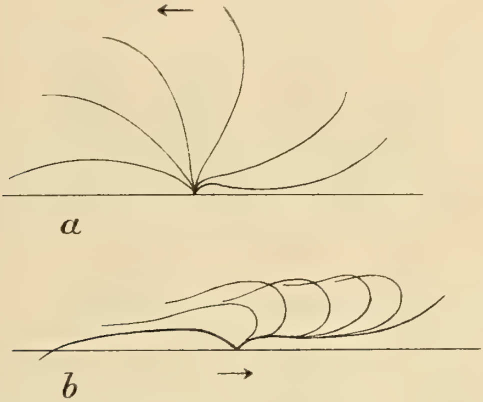

Fig. 3.—Diagram illustrating the form of the terminal cilia of *Mytilus* during (*a*) the effective and (*b*) recovery beats.

It should be mentioned that the effective stroke is always quicker than the recovery stroke. When movement is very rapid it is impossible to see the cilium during the effective beat.

The change in the consistency of the cilium during the two phases of its beat appears to be an observation of considerable significance, but does not appear to have been commented on by other observers. The illustrations given in Verworn's (31) text-book of the cilia of *Urostyla grandis* appear to indicate the same phenomenon. The only detailed description of the movement of large cilia is that by Williams (32) of the cilia of a molluscan larva, which clearly indicates a difference in the elastic properties of the cilia during the two phases of the beat (fig. 4).

The effect of stimulation on a muscle fibre has been compared by Bayliss (1) to the conversion of a stretched lead spring to a stretched steel spring, so that the excited fibre is capable of expending energy in the form of work. The cilia of *Mytilus,* and to a still greater extent the cilia of Ctenophores, can be compared, with equal justice, to bent strips of lead and steel wire. *It seems fairly certain that the energy which is expended by the cilium is stored as tension energy.*

Let us now consider the point of origin of the stimulus to which the movement of the cilium is the mechanical response. The cilia on adjacent cells of the lateral epithelium beat in a definite sequence. If, however, individual cells are separated experimentally, they continue to exhibit active and prolonged movement (Gray, 12). All attempts to detect the operation of nervous elements in the epithelium, or in the cells themselves, have failed. It may be concluded, therefore, that these ciliated cells provide an example of an automatically contractile tissue. The cells are comparable to cardiac muscle cells; each cell is capable of independent movement, although, under normal circumstances, there is a definite co-ordination between adjacent cells.

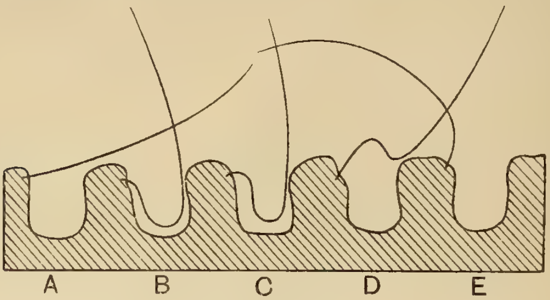

Fig. 4.—Diagram showing successive stages in the stroke of a cilium on the velum of a gastropod larva (after Williams). A, position of rest; B, position at end of recovery beat; C and D, stages during effective beat; E, end of effective beat.

When a piece of living *Mytilus* gill is teased in sea-water under the microscope, portions of the cuticular layer with attached cilia are often stripped away from the cells themselves. Such cilia are invariably motionless. It seems certain, then, that *an essential part of the mechanism lies in the cell itself;* as long as there is a small portion of normal protoplasm at the distal end of the cell near the base of the cilium, the latter continues to move.*

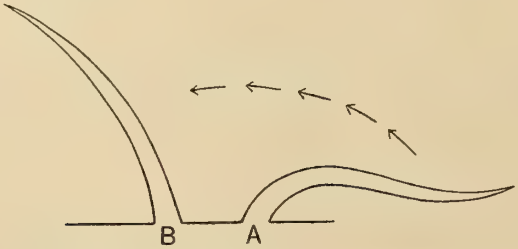

Fig. 5.—Diagram of cilia of *Pleurobrachia.* A is the position of rest; B is the end of the effective stroke, whose direction is shown by the arrows.

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* R. S. Lillie observed that detached but active cilia from the larva of Polygordius possessed a knob-like expansion at their proximal ends.

It is interesting to mention a few experiments which were performed on the ciliated plates of the Ctenophore *Pleurobrachia* (see fig. 5).

Like the lateral cilia of *Mytilus* there is here definite metachronial movement, but the movement of each cell is dependent upon a stimulus passing to it from the cell next to it. If the ciliated comb is cut, then all those cilia situated on the oral side of the cut cease to beat, and come to rest at the beginning of the effective beat. Any cell of this oral portion can be thrown into motion by stimulating the cell immediately above it. As far as one can see, these cilia resemble skeletal muscle rather than cardiac muscle, since some form of external stimulus is necessary to produce a mechanical response from the individual cells.

II. THE INFLUENCE OF THE ENVIRONMENT ON CILIARY ACTIVITY.

It has already been shown (Gray, 12) that a satisfactory medium for ciliary activity is provided by a Van't Hoff's solution containing NaCl, KCl, $MgCl_2$ and $CaCl_2$, in the same proportions as in sea-water, and whose hydrogen ion concentration is about P_H 7·8. We can therefore regard the other constituents of normal sea-water as unessential.

(a) *The Effect of Varying the Hydrogen-ion Concentration.*

The fact that the cilia on the gill of *Mytilus* cease to move when the hydrogen ion concentration of the surrounding medium reaches a limiting value, and that the cilia will resume their movements when the acidity of the medium is reduced (Gray, 12), led to the following investigation of Bernstein's well known hypothesis.

If the hypothesis be sound, then the inhibiting powers of an acid medium should be inversely proportional to the rate at which the acid can enter the cell. It has been shown that mineral acids enter living cells very slowly, if at all, whereas the fatty acids enter readily. Pieces of living gill, stained with neutral red, undergo no change in colour when placed in Van't Hoff solution, to which sufficient hydrochloric acid has been added to produce a hydrogen ion concentration of P_H 3·4: when placed in a similar solution, made acid with acetic or butyric acid, the gill instantly changes from a dull brick red colour to one of brilliant red—indicating that the acid has entered the cells. Corresponding facts apply to the alkalies: ammonia rapidly enters a living cell, whereas sodium hydroxide does not.

It is obvious, therefore, that a method is available for deciding whether the cessation of movement of cilia in an acid medium is due to an interference with the electrical properties of the cell surface, or whether the affected elements lie within the cell itself.

The procedure adopted was to determine the critical concentration of hydrogen ions in the external medium, which was just sufficient to cause complete stoppage of ciliary movement in 1 minute. A very large number of experiments were performed, whose results are summarised in the following Table :—

Acid.	Critical P_H concentration.	Acid.	Critical P_H concentration.
Hydrochloric	3·4	Oxalic	3·1
Sulphuric	3·1	Formic	4·0
Nitric	3·4	Acetic	4·8
Citric	3·4	Butyric	5·2

It will be observed that the mineral acids are of practically uniform efficiency, and the same hydrogen ion concentration of each is required to produce the same physiological effect. The fatty acids, on the other hand, form a series which is more efficient than the mineral acids, and the higher member of the series is distinctly more efficient than a lower member.

It has already been shown (Gray, 12) that, when ciliary activity has ceased in the presence of an acid, recovery takes place when the surrounding medium is made alkaline, so that it is possible to compare the effects of the weak and the strong alkalies as restoratives.

When movement has been stopped by means of a fatty acid, the cells rapidly recover in a solution which is not more alkaline than normal sea-water (P_H 7·8): by means of gill fragments stained with neutral red, this recovery can be seen to be due to the rapid rate at which the acid is removed from the cell. If, however, the cilia have been stopped by a mineral acid, recovery in normal sea-water is relatively slow (Gray, 12), so that such fragments form satisfactory material for testing the restorative powers of the various alkalies. In the following experiments, fragments of the same gill were exposed to a definite strength of mineral acid (HCl, P_H 3·3) for a definite period (5 minutes). Individual fragments were then transferred to normal sea-water, and to sea-water whose P_H had been raised to the same abnormally high value by NaOH and by NH_4OH respectively.

	Sea-water. P_H 7·8.	Sea-water. + NaOH. P_H 8·4.	Sea-water. + NH_4OH. P_H 8·4.
Movement began	12′	7′	1′
Full recovery	25′	19′	3′

The comparative efficiency of ammonia and sodium hydroxide is also seen from the following experiment. Fragments, after previous acid treatment, were transferred to sea-water whose alkalinity had been raised to a known value by NaOH and by NH_4OH.

P_H.	Time in minutes for full recovery in	
	NH_4OH.	NaOH.
9·5	½	5
9·2	½	7
9·0	½	8
8·7	1	8–10
8·5	3	10–12
8·4	5	12–15

The above figures all apply to fragments of the same gill after precisely the same acid treatment. The experiment was repeated several times with identical results.

It is therefore clear that *the weak acids which enter the cell are more efficient inhibitors of ciliary movement than are the strong acids which do not enter readily, and conversely the weak alkalies are much more efficient restoratives than the strong alkalies.*

It is impossible to accept the suggestion that the normal activity of the ciliated cells is upset by acids owing to a disturbance of the cell surface. The physiological effects of both acids and alkalies depend upon the ease with which these reagents penetrate to the cell interior. It may be noted that another series of experiments showed that the presence of neutral electrolytes in external medium has but little effect upon the efficiency of either acids or alkalies.

It is important to note that *the cilia come to rest in an acid solution by a gradual diminution in the rate of the whole beat, without any reduction in its amplitude.* Both the effective and the recovery strokes become slower, and there is often a marked pause at the beginning and end of each stroke, so that a complete beat may take as long as 10 seconds. It is difficult to imagine how this could occur where there is actual derangement of the contractile elements—since we would expect such to be attended by a gradual reduction in the amplitude of the beat.

Again, the cilia invariably come to rest at the end of the effective stroke; that is, in that position in which the cilium itself possesses no available potential energy.

Interesting evidence is available from a study of spermatozoa which are known to possess a limited amount of reserve chemical energy. The effect of acid on such cells is precisely similar to that on ciliated cells (Gray, 13).

Cohn (5) has shown that when the movement of such spermatozoa has ceased in an acid medium, there is no loss of energy,* the conversion of chemical into potential energy has ceased, and can be switched on again by removing the acid from the external environment. *It seems highly probable, therefore, that the movement of cilia is stopped in an acid medium because there is no longer a conversion of chemical into potential energy.*

(b) *Metallic Ions.*

We have already seen that the cilia of *Mytilus* beat normally in an artificial solution containing NaCl, KCl, $CaCl_2$ and $MgCl_2$, and whose hydrogen ion concentration is the same as sea-water. On the whole, ciliary activity is remarkably independent on the absolute concentration of any particular ion or upon the exact ratio between different ions. In an investigation of the effects of individual ions it is necessary to maintain the normal hydrogen ion concentration and also the concentration of other ions in the solution. Further, in making a comparison between the effects of an ion on ciliary and on muscular activity, comparison must be made to a *spontaneously contractile* muscle (*e.g.*, the auricle of the heart). These facts explain the difference in the conclusions arrived at in this paper, and those of Lillie (20), Höber (16) and Mayer (23).

Experiments with Potassium Chloride.—If KCl be omitted from the external medium and its place taken by NaCl, the *lateral cilia* come to rest. The time taken for the movement to cease varies considerably in different gills. In most cases movement slows down after 5–10 minutes, and in less than 20 minutes the cilia are stationary. In a few cases in which the lateral cilia showed very active movement before the experiment, movement was continued in the absence of potassium for as long as 45–60 minutes; movement recommences vigorously on the subsequent addition of potassium, or on the addition of a small amount of alkali sufficient to raise the P_H to about 8·5. It should be mentioned that in several cases the lateral cilia ceased to beat in normal sea-water after about $1\frac{1}{2}$ hours, but on addition of a slight amount of KCl, vigorous movement took place. This may possibly be due to the fact that the blood of the animal contains a higher concentration of potassium than does normal sea-water, so that when isolated in sea-water the gill is in an environment which may have a sub-normal concentration of potassium.

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* E. G. Martin (22) found that, in the presence of alkali, spontaneously beating strips of the ventricle of the tortoise gave out a constant total amount of energy for a unit mass of tissue.

In contrast to the lateral cilia, the fronto-lateral, the frontal and the terminal cilia beat normally for very long periods (more than 48 hours) in a solution containing no potassium.

This contrast is paralleled by the action of such a solution when perfused through the heart of different molluscs. In the case of *Pecten* Mines (25) showed that potassium could be omitted from the perfusion fluid without any derangement of the heart beat: on the other hand, the heart of the *Octopus* gradually stops in such a solution (Kleefeld, 17), and can be revived on adding potassium.

If potassium is present in excess a similar contrast is found in its effects on the different cilia. Until the concentration of potassium is raised to about ten times the normal value, little or no effect is noticeable upon the cilia, although there is a tendency for a rapid secretion of mucus of the surface of the gill, which may clog the frontal and terminal cilia. Above this concentration the *fronto-lateral* cilia are affected in a curious way—they pass into a state of contraction which persists for a considerable period. At first the tips of the cilia remain bent at the end of the recovery phase of the beat, then a wave passes along a whole series of the cilia which accentuates this bend to a marked degree; this is followed by another wave in which one cilium after another remains fixed in a completely contracted position (*i.e.*, at the end of the effective beat). In this position the cilia often exhibit a curious quivering movement.

There is thus a regular "staircase" effect—similar to that found in the case of the heart.

If the concentration of potassium has not been too strong, the cilia recover on transference to normal sea-water after about 15 minutes. Even if contraction of the fronto-lateral cilia is brought about by a solution in which the whole of the NaCl in the Van't Hoff solution has been replaced by KCl, it is noticeable that after about 45 minutes, the cilia begin to recover in the original solution, the amplitude of the beat getting gradually larger until the complete beat is resumed. The rate of the recovery in such a solution, or in normal sea-water, is greatly hastened by the presence of alkali.

In all solutions containing excess of KCl, the beat and rhythm of the lateral cilia is well maintained and is often more rapid than in the normal gill; the frontal and terminal cilia are either unaffected or beat more rapidly than normal. Here again the differential action of potassium on different tissues is clearly illustrated.

Lillie and Höber have both emphasized the maintenance of ciliary movement in the presence of excess of potassium, and have contrasted this with the depressant effect of such an excess on skeletal muscle. In view of the effect of KCl on the fronto-lateral cilia in preventing the recovery beat, it is interesting to note that this is also its effect upon skeletal muscle, viz., the latter is thrown into a state of prolonged contraction (Mines); the same thing occurs in the vertebrate heart (Burridge, 3).

The recent work of Kolm and Pick (18) on the effect of potassium on the heart brings out clearly three points: (i) the marked quickening effect on the automatically contractile auricles and sinus; (ii) the differential action on different tissues in the same organ, viz., auricles, ventricle; (iii) the prolonged contraction which is caused by high concentration of potassium: the contraction eventually passing off in the presence of the same perfusion fluid.

As far as I am aware there is no evidence against the view that in the case of automatically contractile tissues the effect of low concentrations of potassium salts is to increase the rate of movement, while stronger concentrations cause a prolonged contraction which is not, however, permanent.

The relative immunity of cilia as compared to a muscle cell to potassium is probably due to the fact that the latent period of the cilium is very much less than that of the average muscle cell, so that an environment which throws the latter into tonic contraction by increasing the rate of activity of the cell, has much less effect on the cilia.

The Effects of the Sodium Ion.—We have already mentioned that the absolute concentration of Na can be raised considerably without deranging ciliary movement. If the concentration of $CaCl_2$, $MgCl_2$, and KCl be kept constant, and the sodium chloride replaced wholly by isotonic saccharose, ciliary movement is well maintained for several hours.

It seems reasonable to conclude therefore that the sodium ion plays no specific *rôle* in activity—although it probably enters into the conditions of the general equilibrium within the cell.

The Effects of the Magnesium Ion.—If magnesium be omitted from the Van't Hoff solution, and its place taken by an appropriate amount of calcium, ciliary action is well maintained for many hours (more than 48 hours). Within wide limits a variation in the concentration of magnesium in the medium has little effect upon the form or rate of beat of the terminal cilia.

This fact is in accordance with observations upon the heart of the Octopus (Fredericq), the arms of Lepas (Mayer), and the heart of Salpa (Mayer)—all of which are insensitive to an absence of magnesium. The sensitivity of the heart of Pecten (Mines) is doubtless correlated with its high sensitivity to hydrogen ions (Mines).

In a subsequent paper, however, evidence will be presented which indicates that magnesium plays an important *rôle* in the economy of the cell.

The Effects of the Calcium Ion.—If calcium be omitted from the external medium, and the other conditions be the same as in normal Van't Hoff solution, prolonged ciliary movement takes place. The effect of the absence of calcium is, however, seen in the increased sensitivity of the cell to hydrogen ions. This is seen in the following experiment:—

P_H.	Duration of movement in	
	Van't Hoff solution, containing calcium.	Van't Hoff solution, without calcium.
7·8	More than 48 hours	More than 48 hours.
7·0	More than 48 hours	15-45 mins.

If, after the cessation of movement in the absence of calcium, the alkalinity of the solution be raised, rapid recovery takes place. If, on the other hand, calcium is added, the amount of recovery, at least for a time, depends upon the time which has elapsed between the cessation of movement, and the addition of the calcium. If the time is short, rapid and complete recovery takes place within 1 minute. If the time be prolonged, the recovery, on addition of calcium, is slow: the amplitude of the beat is regained almost at once, but the rate of both the effective and recovery strokes is slow, and there are often marked pauses at the beginning and end of each stroke. The whole phenomenon recalls the effect of acid, and one might conclude that the effect of the absence of calcium is possibly due to a change in the cell produced by a change in the hydrogen ion concentration.

The cessation of movement in the absence of calcium, and the recovery of movement on the addition of calcium or hydroxyl ions, is paralleled by the reaction of such solutions on the heart.

(c) *The Effect of varying the Osmotic Pressure of the Surrounding Medium.*

Although the cilia are not sensitive to slight changes in osmotic pressure, yet, if this exceeds a certain value, the cilia are rapidly brought to a complete standstill. On reducing the osmotic pressure, instant and complete recovery takes place. These facts are extremely easy to demonstrate, and can be repeated a great many times with the same piece of gill.

It does not matter whether the increase in osmotic pressure is brought about by the addition to sea-water of non-electrolytes or by balanced electrolytes (*e.g.*, $2\frac{1}{2}$ M. Van't Hoff solution). In a solution which is not quite strong enough to cause complete stoppage, it is noticeable that a reduced movement is maintained for a very long time (more than 24 hours); in such a solution the amplitude of the beat is much reduced, whereas the rate of beat is almost unaffected. In a solution which is strong enough to cause complete stoppage, the cilia remain in the position shown in the accompanying diagram (fig. 6), so that they are unusually conspicuous.

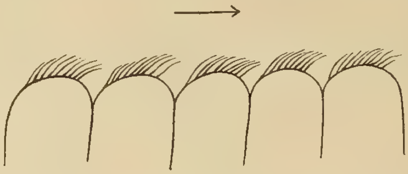

Fig. 6.—Diagram illustrating the appearance of the terminal cilia of *Mytilus* when brought to rest by high osmotic pressure of the external medium. The arrow shows the direction of the normal effective beat.

It is important to notice that the stoppage of the cilia in hypertonic solutions is brought about in an entirely different way to the stoppage in an acid solution, and it is therefore not surprising to find that the stoppage in hypertonic solutions is not influenced by the presence of hydroxyl ions, nor is the effect of an acid solution altered by reducing its osmotic pressure.

The effect of hypertonic solutions on muscular activity has not been extensively studied, but Demoor and Phillipson (7) have shown that the skeletal muscles of a frog lose their excitability to a direct stimulus when immersed in hypertonic Ringer solution; the muscles shorten somewhat, and the response is gradually abolished. These effects are entirely reversed by treatment with normal Ringer solution. Carlson (4) also found that the *rate* of beat of the auricles of the tortoise is unaffected by perfusion with hypertonic Ringer solution; the *amplitude* was, however, much reduced—and recovered in normal Ringer.

The fact that the amplitude of the beat is affected by an increase in the osmotic pressure of the external medium seems to indicate that a loss of water from the cell interferes not with the periodic liberation of energy, but with the actual contractile mechanism.

IV. SUMMARY AND DISCUSSION.

Let us now review the whole of the available facts, and attempt to form a working hypothesis of the nature of the ciliary mechanism. We have seen that the ciliated cells of *Mytilus* provide an example of an automatically contractile tissue; they differ from cardiac or smooth muscle in that their latent period is extremely short, and the rate of beat very much quicker than corresponding muscle cell. The cilium is essentially an elastic fibre in communication with and dependent upon the protoplasm of the cell.* The cilium is capable of storing potential energy (supplied to it from the cell) in the form of tension, and of liberating this energy in the form of work. The amount of tension developed depends on the existence of free water in the cell. The rate at which the energy is stored and liberated by the cilium depends upon the hydrogen ion concentration of the cell interior. Whereas the rate of movement of the cilium depends almost completely upon the concentration of hydrogen ions inside the cell, it is largely independent of the presence of specific metallic ions (except in certain cases potassium) in the external medium.

A suggestion as to the way in which chemical activity within the cell may lead to the development of a tension in a fibrous structure (in the presence of water) is provided by the experiments of Fischer and Strietmann (9). These authors have shown that, if a piece of catgut, suspended in water, comes into contact with an acid, the fibre absorbs water and develops a considerable tension. The same phenomenon occurs in the presence of an alkali. In this experiment the essential conditions are: (i) the liberation of a chemical (acid or alkali) on the surface of a fibre; (ii) the presence of water. These facts form the basis of an hypothesis of muscular actions, but they can be applied with equal force to the ciliary mechanism. In the case of the muscle fibre, the production of an acid (lactic acid) during stimulation has been demonstrated. If the same fact be assumed to be true in the case of a cilium, then many of the facts stated in this paper receive a reasonable explanation. The rate at which lactic acid is produced from its carbohydrate precursor depends upon the hydrogen ion concentration of the medium (Kondo, 19). Hence, the rate at which chemical energy can be converted into potential energy will also depend upon the hydrogen ion concentration of the cell interior. It is therefore clear why the rate of the recovery stroke of the cilia of *Mytilus* is affected by acids which enter the cell. By our hypothesis, at the end of the recovery stroke the cilium possesses potential energy, owing to the stress set up in its elastic structure by the tension of those fibres at whose surface an acid is situated. This potential energy can only be liberated by the relaxation of the fibres; that is, by the removal of the acid. The rate at which the acid is removed will depend upon the degree of alkalinity of the surrounding cell contents. In other words, an explanation is available for the effect of acids and alkalies on the rate of the effective stroke of the cilium.

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* The truth of this statement can be seen by reference to most text-books of histology, or to the work of Saguchi (29).

The alteration in the length or tension of a fibre exposed to acid depends upon the concentration of salts present (Fischer), or on the ease with which water can be drawn from the surrounding fluid. Hence, when ciliated cells are exposed to a solution whose osmotic pressure is capable of withdrawing a considerable amount of water from the cells, the amount of tension, set up by a normal amount of acid at the surface of the fibres, will be reduced; consequently, in solutions of high osmotic pressure, the amplitude of the beat is affected, and the cilia stop when the amount of free water is zero.

The above conception of the ciliary mechanism has two advantages: (i) it does not endow the cilium with any hypothetical structure; (ii) it brings the mechanism into line with what is known of other contractile tissues. There is, however, one corollary to the hypothesis which applies equally to cilia and to muscle cells. If the liberation of an acid at the surface of the ciliary fibrils enables the cilium to store potential energy and perform the recovery stroke—then, when ciliary activity ceases in the presence of an experimental acid, we must assume that the latter acid does not come into contact with the contractile fibrils, since the cilia come to rest with the fibrils relaxed. When, however, stronger concentrations of acid are used, the cilia stop partly (in some cases almost completely) contracted, and occupy a position near the end of the normal recovery stroke (see fig. 7). We may well suppose that in this case the experimental acid has reached the contractile fibres. Precisely the same phenomena are found in the heart: weak

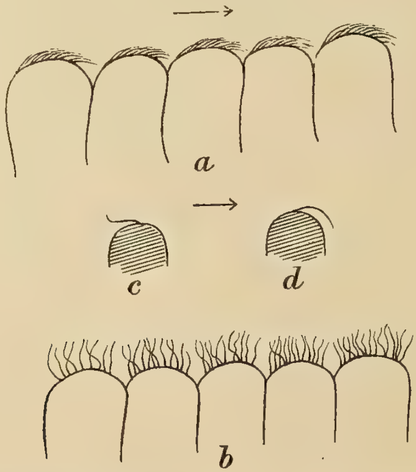

Fig. 7.—Diagram illustrating the effect of acid on the terminal cilia of *Mytilus*. (*a*) With acid just strong enough to stop cilia; note all the cilia stop at the end of the effective beat. (*b*) Acid of considerably greater strength; note the cilia come to rest between the two phases of the recovery stroke. (*c*) and (*d*) Positions at beginning and end of the normal effective stroke.

concentrations of acid stop the heart in diastole, stronger concentrations stop it in systole.

Finally, if the cilia of *Pleurobrachia* are considered, it will be obvious how very closely the known facts agree with the hypothesis of muscular action outlined by Hill and Hartree (15). The position of rest of these cilia is at the end of the recovery stroke, so that (like a striated muscle fibre) they possess a definite amount of potential energy, which can be released at the moment of stimulation. The cilium may be regarded as a series of fibres, B,* which are in communication with a network, A; the walls of the latter are kept stretched by the presence of water in the interstices of the network. It is this turgidity which provides the cell with the potential energy possessed at the position of rest. At the moment of stimulation, some chemical substance is set free at the surface of the fibres, B, which promptly take up water from the network, A, so that the cilium flies forward, owing to: (i) the liberation of the energy stored in the walls of A; and (ii) the tension developed in B. At the end of the effective stroke, the chemical substance is removed from the fibres, B, and the water flows away from the fibres (*e.g.*, by osmosis) into the interstices of the network, A, thereby stretching the cilium back to the resting position.

It should be understood that the above analysis of the ciliary mechanism is nothing more than a working hypothesis. At the same time the remarkable similarity between the conditions necessary for ciliary and muscular activity, coupled with the apparent similarity in the fibrous structure of the two types of cell, leads to the conclusion that the two mechanisms may be essentially similar. It is also clear that the same scheme might be applied to pseudopodial movement.

Summary of Experimental Results.

1. The cilium is capable of expending potential energy in the form of work as long as it is in organic connection with the cell protoplasm.

2. Each ciliated cell of *Mytilus* is capable of independent movement when isolated. The cilia of the Ctenophore *Pleurobrachia* require a definite stimulus to induce their beat. Both types of cilia show metachronial rhythm.

3. The cilium is an elastic fibre or bundle of fibres. In the large majority of cases the cilia are in communication with the cell protoplasm by means of intracellular fibrillæ.

4. The ciliary beat consists of a rapid effective stroke and a slower recovery stroke. The form of the recovery stroke often differs markedly from the effective stroke.

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* The fibrous nature of these cilia is well seen in preserved specimens.

During the former the cilium resembles a slack string or fibre, whereas during the effective stroke its rigidity is distinctly greater.

5. When exposed to an acid solution of appropriate strength the cilia of Mytilus come to rest by a gradual slowing of the whole beat: the amplitude of the beat is not affected. The cilia always come to rest at the end of the effective stroke, *i.e.*, in the position in which the cilium possesses no convertible potential energy.

The cessation of movement in an acid solution is due to a change which takes place inside the cell, and not at its surface. Evidence is advanced which suggests that the presence of acid prevents the conversion of chemical energy into kinetic energy.

The effect of acid is entirely reversible by alkalies. The rate of the beat is most simply controlled by controlling the hydrogen ion concentration within the cell; up to a certain point the higher the internal alkalinity the more rapid is the ciliary beat.

6. Under normal circumstances the activity of the *lateral* cilia depends on the presence of potassium ions. This effect is probably due to the general effect of the ion in quickening the beat, which leads in the case of the *fronto-lateral* cilia to a state of prolonged contraction when potassium is in excess.

7. Ciliary activity is not sensitive to change in the concentration of magnesium or sodium in the external medium, although these ions play a part in the general equilibrium between the cell and its environment.

8. The absence of calcium ions may bring about a cessation of ciliary movement, which is antagonised by hydroxyl ions.

9. The reaction of cilia and of muscles to the various chemical constituents of their environment is essentially the same. The apparent differences are due to: (i) the greater sensitivity of most muscles as compared to cilia; (ii) the cilia have a much shorter latent period than most muscles; (iii) a ciliated cell cannot be regarded as directly comparable to a neuro-muscular system.

10. Cilia are brought to rest if the osmotic pressure of the external medium exceeds a certain value. The stoppage is brought about by a gradual reduction in the amplitude of the beat. These effects are entirely removed on reducing the osmotic pressure.

11. An hypothesis is put forward that the mechanism of ciliary movement is essentially the same as that of muscular movement.

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The Mechanism of Ciliary Movement. II.—*The Effect of Ions on the Cell Membrane.*

By J. Gray, M.A., Fellow of King's College, Cambridge, and Balfour Student in the University of Cambridge.

(Communicated by Prof. J. S. Gardiner, F.R.S. Received October 7, 1921.)

In 1906 R. S. Lillie (7) published an account of the effects of various pure sodium salts upon the ciliated epithelium of *Mytilus edulis.* He found that the ions could be arranged in the following order of "toxicity,"

$$Cl' < NO_3' < Br' < I' < SCN'.$$

Lillie also referred to the effect which various anions have upon the amount of water taken up by the cells from their external medium, and arranged the ions in the following order of efficiency in causing an absorption of water by the cells.

$$CH_3COO' < Cl' < NO_3' < ClO_3' < Br' < I' < SCN' < BrO_3' < OH'.$$

These results form the basis of the following statements found in recent text-books, viz., Höber (6) and Bechhold (1):—

(i) That the above series represents the effect of anions on ciliary movement.

(ii) That the order in which anions affect ciliary movement is the reverse of that in which they affect muscular movement.

One of the objects of the present communication is to consider whether these important statements are justified.

It has already been shown (Gray (3)), that if the ciliated epithelium of *Mytilus edulis* is placed in a solution containing NaCl, KCl, $CaCl_2$ and $MgCl_2$, the whole tissue remains normal and in activity for a very prolonged period. If we wish, therefore, to determine what specific *rôle,* if any, is being played by the chlorine ion, it is necessary to replace this ion in the above solution by other anions. It is not permissible to use a solution which does not contain either $K^{\cdot}$, $Ca^{\cdot\cdot}$, or $Mg^{\cdot\cdot}$, nor is it permissible to ignore the hydrogen ion concentrations of any experimental solution (Gray (3)).

In the experiments tabulated in Table I, each solution contained the same molecular concentration of cations, and the hydrogen ion concentration was kept well within the limits to which the tissue is indifferent.

Table I.—The Effect of Mixtures of $Na^{\cdot}$, $K^{\cdot}$, $Ca^{\cdot\cdot}$, and $Mg^{\cdot\cdot}$ upon the Movement of the Terminal Cilia of *Mytilus*.

Anion.	Time.					PH.
	30 mins.	1 hour.	2 hours.	3 hours.	5 hours; exp. discontinued.	
Chlorides ……	Normal movement; no absorption of water.	Normal movement; no absorption of water.				7·8
Nitrates ………	Normal movement; no absorption of water					7·8
Iodides ………	Very rapid movement; no absorption of water.			Normal movement; no absorption of water.		8·0
Bromides ……	Normal movement; no absorption of water					7·8
Acetates ………	Normal movement; no absorption of water					7·9
Sulphates ……	Very rapid movement; no absorption of water.		Normal movement; no absorption of water.			8·0
Tartrates ……	Normal movement.	Slow movement.	Very slow movement.	No movement		7·8
			No absorption of water.			
Citrates ………	No movement; no absorption of water					7·8

Such experiments shew quite clearly that in a solution containing balanced cations, the substitution of the normal anion chlorine by NO_3', I′, Br′, Ac′, or SO_4'' is not attended by any interference with either the activity of the cilia or the amount of water taken up by the cells. It is possible that in the case of the iodide and sulphate mixtures there is an actual increase in the rate of beat of the cilia, although the observed effects may possibly be due to the slightly higher hydroxyl ion concentration of these solutions.

In considering the possible significance of the cessation of movement in balanced solutions of tartrates and citrates, we are confronted with a purely chemical problem. Calcium tartrate and calcium citrate are both very sparingly soluble in water; the same statement applies, though to a less degree, to the magnesium salts. When, however, these salts are added to a solution of the corresponding sodium salt, they dissolve to a very marked extent. The suspicion arises that in making up a physiological mixture of tartrates or citrates, the metals magnesium and calcium are not present as free ions. This suspicion seems justified by the following facts. When sodium phosphate and ammonia are added to a saturated solution of magnesium citrate in water, a definite precipitate of magnesium phosphate is formed. If, however, sodium phosphate and ammonia are added to a solution of magnesium citrate in sodium citrate, no precipitate is formed, although a considerable amount of magnesium is in solution. We may conclude, therefore, that the inability of tartrate or citrate mixtures to maintain the normal equilibrium of ciliated cells may prove to be due to the absence of free magnesium and calcium ions and not to the direct effect of the anions. Confirmation of this view will be given later in this paper.

As far as I am aware, the only satisfactory investigation of the effects of anions on other living processes is that of Sakai (9) on the heart of the frog. This author used solutions containing balanced cations, but does not refer to their hydrogen ion concentration. His results may be summarised as follows:—

I', Br', NO_3'	Beat well maintained, even faster than normal.
Cl'	Normal.
SO_4''.........	Beat well maintained, after initial slowing.
Cit'''	Rate steadily falls and finally heart stops.

There is clearly no fundamental difference between the effect of anions on ciliary and on muscular activity. Both types of tissue are remarkably indifferent to wide variations in the nature of the anions in the external environment.

(b) *The Effects of Solutions of Pure Sodium Salts.*

Whereas in a solution of a sodium salt containing $K^{\cdot}$, $Ca^{\cdot\cdot}$, and $Mg^{\cdot\cdot}$, the ciliated epithelium is remarkably indifferent to a variation in the nature of the anions present, yet in a solution of a sodium salt, which does not contain other cations, the tissue shows a very marked sensitivity to particular anions. Table II shows the general course of events.

It will be seen that the salts can be divided into two main groups:

A. Those salts which cause the cells to swell up by the absorption of water. $Cl' <$, $NO_3' <$, $Br' <$, I';

B. Those salts which do not cause the cells to swell. SO_4'', Tartrate, Citrate;

while the acetate forms an intermediary type.

It is also clear that these solutions cannot indicate the direct effect of the environment on the contractile mechanism; their effect on the cell is of a more general nature, and the contractile mechanism is only secondarily involved.*

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* Cilia often remain active when the process of absorbing water is relatively far advanced.

Table II.—The Effect of Pure Sodium Salt Solutions upon Ciliated Epithelia.

Anion.	Time.					P_H.
	30 mins.	1 hour.	2 hours.	3 hours.	5 hours.	
Chloride............	Some cilia active. A little swelling.	Cells considerably swollen. Some cilia destroyed.	Tissue disorganised. Cells much swollen. Cilia destroyed.			7·8
Nitrate	A few cilia active. Most cells begin to swell.	Cells much swollen. Few cilia left.	Tissue disorganised. Cells much swollen. Cilia destroyed.			7·8
Bromide............	A few cilia active. Most cells begin to swell.	Cells much swollen. Few cilia left.	Tissue disorganised. Cells much swollen. Cilia destroyed.			7·8
Iodide	Nearly all cells markedly swollen.	Tissue disorganised. Cells much swollen. Cilia destroyed.				8·0
Acetate	No movement. Cells normal.	No movement. Cells slightly swollen				7·9
Sulphate	No movement: cells do not swell. Cilia remain healthy in appearance.					8·0
Tartrate	No movement: cells do not swell. Cilia remain healthy in appearance.					7·8
Citrate	No movement: cells do not swell. Cilia remain healthy in appearance.					7·8

The effects of the various sodium salts upon the water-content of the cells is precisely what one would expect from the effects of the same salts on the water-content of such non-living colloid gels as fibrin or gelatine. Such gels swell readily in the presence of hydroxyl ions, but the uptake of water is affected by different salts in precisely the same way as we have found for ciliated cells.

Now, during life the interior of the cell is always more acid than the external medium; although at the same time it must be remembered that the cell colloids are on the alkaline side of their isoelectric point. Hence, if the cell interior is allowed to come into contact with the external medium, the amount of water taken up at any particular hydrogen ion concentration will depend upon the nature of the anions present in the medium. This is seen above actually to be the case, when the cells are put into pure solutions of sodium salts. It is not the case when the cells are in a solution containing balanced cations. In the latter type of solution the hydroxyl ions outside the cell cannot penetrate the cell, nor can the anions outside the cell exert any influence upon the uptake of water by the cell. Such a condition of affairs is obviously due to the semi-permeable nature of the normal cell membrane. The inference to be drawn is, of course, that when placed in a solution of a pure sodium salt the observed effects are the direct outcome of the loss of semi-permeability by the cell membrane. Direct proof of this statement is provided by a determination of the electrical conductivity of normal tissues placed in pure sodium salts. Osterhout (8) showed that the electrical resistance of *Laminaria* tissue placed in sodium chloride solution fell steadily, until it was one-third of its original value; Gray (4) showed that sodium citrate caused a marked fall in the resistance of Echinoderm eggs, and quite recently Shearer (11) has shown the similar effects of sodium chloride on bacteria.

(c) *The Nature of the Cell Membrane.*

Lillie (7) showed that the toxic effect of pure sodium salts is prevented by the presence of the alkaline earth metals. This has been confirmed and the statement can be somewhat enlarged. In the first place, either magnesium or calcium can prevent the destructive action of a pure sodium chloride solution upon the cell-membrane; or, as is perhaps the more correct mode of expression, the presence of magnesium or calcium is necessary for the semi-permeable properties of the cell membrane. Since both calcium and magnesium occur in sea-water, it is of importance to determine, if possible, which of these two metals maintains the normal stability of the cell surface.

In the following experiment the concentration of each salt was the concentration in which it normally occurs in sea-water.*

$NaCl$	P_H 7·0	Complete disintegration after 3 hours.
$NaCl + CaCl_2$	P_H 7·0	No disintegration after 24 hours. Some cilia destroyed, and some filaments separated from their neighbours.
$NaCl + MgCl_2$	P_H 7·0	Tissue quite healthy, but cilia motionless after 24 hours.

It is clear that the amount of magnesium in normal sea-water is alone capable of maintaining the cell-surface: at the same time the amount of calcium is also sufficient to produce a very well-marked stabilising influence. In the solution which contains only calcium and sodium in their normal concentrations, the tissue does not remain so healthy as in that containing magnesium and sodium; in the former solution there is a distinct tendency for the finer cilia to become opaque and detached from the cell: this is particularly the case with the small cilia on the ciliated junctions, so that individual gill filaments show a distinct tendency to separate from each other (see Gray (3)). Interesting facts concerning the action of calcium are, however, available from a study of cells whose normal semi-permeability has been destroyed by immersion in those sodium salts which prevent the uptake of an abnormal amount of water by the cell. When ciliary movement has ceased in sodium citrate, the cilia and the tissue appear quite healthy and translucent. On transference to normal sea-water, however, the cilia at once become opaque, and are completely destroyed; at the same time there is a rapid uptake of water by the cells which swell up in the usual way. If, on the other hand, the tissue (after initial citrate treatment) be placed in sea-water containing no calcium, the cells and cilia remain quite healthy, and complete recovery of movement takes place, although the rate of beat is usually slower than the normal; after a time the rate of beat falls off, but can be revived permanently by adding calcium. The same recovery from sodium citrate treatment can be effected by treating the tissue with any solution containing magnesium but no calcium. The same experiments can be performed with tissues previously treated with sodium sulphate or sodium tartrate. It is clear from numerous experiments that magnesium is the only ion in sea-water which will re-form a semi-permeable membrane round a cell which has lost this structure by exposure to a pure sodium salt.

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* The composition of the sea-water used was :—$NaCl$, 28·3 grs. per litre ; KCl, 0·76 grs. per litre ; $MgCl_2$, 5·01 grs. per litre ; $CaCl_2$, 1·22 grs. per litre.

At this point it is interesting to note that calcium has a double action on ciliated cells: (*a*) It is capable of maintaining the cell surface in a normal state of semi-permeability; (*b*) it is necessary for continuous movement in a solution of P_H 7·0 (see Gray (5)). In the first of these functions calcium can be completely replaced by magnesium, but cannot be so replaced in the second.

The destructive effect of sodium salts upon the cell membrane is shared by other monovalent cations, although to a variable extent. In the case of the chlorides, there is little difference between $Na^{\cdot}$, $NH_4^{\cdot}$, and $K^{\cdot}$, while the effect of $Li^{\cdot}$ is considerably less. In the case of other salts, *e.g.*, tartrates and citrates, the erosive power of the potassium salts appears to be distinctly less than that of the corresponding salts of sodium. Pure solutions of magnesium and calcium salts have no erosive action for a considerable time (six to eight hours), after which the cell membranes begin to be affected.

It is curious to notice that magnesium has little or no stabilising action against the erosive power of potassium, whereas calcium has a well-marked action. It is conceivable that this fact may ultimately be correlated with the observation that calcium and potassium are mutually inhibitory in the case of certain tissues; also, if the cell surface be normally maintained by magnesium, then it is possible that potassium may be able to penetrate under normal conditions whereas sodium does not.

Since we can control the destructive effects of the monovalent salts by means of magnesium and calcium, it is interesting to note that the direct action of the different monovalent ions upon the ciliary mechanism forms a well-marked series.

In the following experiment the monovalent salts were present in the molecular concentration of the sodium in normal sea-water; the divalent salts were in the same concentration as they normally occur in sea-water:—

Solution.	P_H.	Movement of terminal cilia.					
		2′.	5′.	10′.	20′.	30′.	60′.
$LiCl$, $MgCl_2$, $CaCl_2$	7·0	0	0	0	0	0	0
$NaCl$, $MgCl_2$, $CaCl_2$	7·2	+ +	+ +	+ +	+ +	+ +	+ +
NH_4Cl, $MgCl_2$, $CaCl_2$	7·0	+ + ⊕	+ + ⊕	+ + ⊕	+ + ⊕	+ + ⊕	+ + ⊕
KCl, $MgCl_2$, $CaCl_2$	7·2	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
Sea-water (HCl)	7·2	+ +	+ +	+ +	+ +	+ +	+ +

0 = no movement.
+ + = normal rate of beat.
+ + ⊕ = quicker than normal.
+ + + = very rapid beat.

Apart, therefore, from their action on the cell-wall, the monovalent ions exert a definite effect on the rate of beat of the cilia. The order in which the various ions increase the rate of beat is well marked, and is as follows:—

$$Li < Na < NH_4 < K.$$

The stoppage of the cilia in the lithium mixture is of the same nature as that observed in acids (Gray (5)), and it is not surprising to find that by raising the alkalinity of the lithium mixture, a rapid and well-maintained beat takes place.

The fact that the presence of potassium induces a more rapid rate of beat than a corresponding amount of sodium enables us to test whether the normal semipermeability of the cell membrane is essential to ciliary activity or not. The experiment can be performed as follows:—Gill fragments are placed in an isotonic solution of sodium citrate until all movement has ceased. Individual pieces are now placed in the following solutions and their behaviour noted.

M/2 NaCl	P_H 7·8	No recovery. Cilia remain healthy, but cells absorb water rapidly.
Sea-water	P_H 7·8	Cilia at once become opaque. Cells rapidly absorb water.
M/2 KCl	P_H 7·8	Marked recovery of beat: recovery is temporary, and cells rapidly swell.

If, whilst the period of recovery in M/2 KCl is well marked, the gills are transferred to sea-water or to any solution containing calcium, the cilia instantly become opaque, and the cells swell rapidly. It is clear, therefore, that although such cilia may beat rapidly in the presence of potassium chloride, yet the semipermeability of the cell-wall has been entirely destroyed by the previous treatment with sodium citrate. The only metal capable of re-stabilising the cell-wall is magnesium.

A further study of this problem obviously leads to a consideration of the nature of the cell-membrane itself, and although our knowledge is very far from complete, interesting analogies may be pointed out in the case of non-living systems. One of the most characteristic features of cell-membranes is their capacity for allowing weak alkalies and acids to pass into the cell and yet exclude the strong alkalies and acids. These facts have, of course, led to the suggestion that the cell-membrane is essentially lipoid in nature—or that, at least, it contains a lipoid phase. Now Clowes (2) has shown that the nature of an oil and water system depends upon the nature of the ions present in the system. The truth of this statement may be very simply verified as follows:—Into five test-tubes are placed 10 c.c. of olive oil containing a little oleic acid, and an equal volume of test solution, together with 0·5 c.c. N/10 NaOH. The tubes are then thoroughly shaken by hand, and examined after about five minutes.

Clowes maintains that a system of water-drops in a continuous phase of oil may be converted into a system of oil-drops in a watery phase by means of sodium chloride. It must be pointed out, however, that grave difficulties attend the suggestion that the surface of the cell possesses a continuous oily phase; nevertheless, these experiments indicate the possible mode of operation of bivalent metals on the cell-surface, if the latter in any way resembles an oily emulsion.

In some ways an even closer analogy to the experiments described in this paper is provided by the experiments of Schryver (10). This author has shown that when a 2 per cent. sodium cholate solution is heated in the presence of calcium a gel is formed. This gel is eroded by solutions of the salts of monovalent cations, but is completely stable when in contact with a solution of balanced monovalent and divalent cations. The erosive power

Table III.

Test tube.	Test solution.	Nature of resulting system.
A	10 c.c. distilled water	A uniform milk-white emulsion of oil drops in water. Discontinuous phase—Oil. Continuous phase—Water.
B	10 c.c. M/2 NaCl	Two phases separate out in equal volumes— (i) Water almost free of oil. (ii) Oil almost free of water.
C	10 c.c. M/10 $MgCl_2$	A uniform greasy emulsion of water drops in oil. Discontinuous phase—Water. Continuous phase—Oil.
D	10 c.c. M/10 $CaCl_2$	Same as C.
E	10 c.c. sea-water	Two phases separate— (i) A large volume of water drops in a continuous oil phase. (ii) A small volume of oil drops in water.

of the monovalent ions depends upon several factors: (*a*) their concentration, (*b*) the salts existing in the gel. In concentrations equivalent to those used in the present series of experiments (viz., about M/2), the erosive power of LiCl is much less than that of Na or K. A study of Schryver's results shows that the analogy between them and the experiments here described is strong. It remains, however, to be proved that such a system as a cholate gel possesses the same semipermeable properties as a living membrane.

Summary.

(i) The ciliated cells of *Mytilus edulis* are insensitive to the following anions:

$$Cl', NO_3', Br', I', Acet', SO_4'',$$

as long as the normal equilibrium of the cations $Na^{\cdot}$, $K^{\cdot}$, $Ca^{\cdot\cdot}$ and $Mg^{\cdot\cdot}$ is maintained in the surrounding medium.

(ii) In solutions containing tartrates and citrates, the bivalent metals $Mg^{\cdot\cdot}$ and $Ca^{\cdot\cdot}$ are probably not present in the ionic state, and the cells behave as though these metals were absent.

(iii) There is no justification for the statement that the order in which anions affect ciliary motion is the reverse of the order in which they affect muscular movement.

(iv) Pure solutions of sodium salts destroy the normal semipermeable nature of the cell-membrane, and the cell colloids behave as an elastic gel in direct contact with the external medium.

(v) The action of sodium salts can be inhibited by magnesium or by calcium. Probably in normal sea-water the stability of the cell-membrane is due to magnesium and not to calcium.

(vi) In balanced solutions the monovalent cations have a direct effect upon the rate of ciliary movement. The rate of movement in solutions of the same hydrogen ion concentration is slowest in lithium and fastest in potassium. The ions can be arranged in the following well-marked series:—

$$\mathrm{Li}^{\cdot},\ \mathrm{Na}^{\cdot},\ \mathrm{NH_4}^{\cdot},\ \mathrm{K}^{\cdot}.$$

(vii) The normal semipermeability of the cell-wall is not an essential condition for ciliary movement.

(viii) The way in which solutions of the different metals affect the cell-surface is in complete agreement with the effect which they produce on the electrical conductivity of the cell.

(ix) There is a marked analogy between the action of salts on the living cell-membrane and on a cholate gel, or oil and water emulsion.

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On the Hypertrophy of the Interstitial Cells in the Testicle of the Guinea-Pig under different Experimental Conditions.

By Alexander Lipschütz, M.D., Professor of Physiology (in collaboration with Benno Ottow, M.D., Charles Wagner, Sc.D., and Felix Bormann).

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(From the Physiological Institute of the University of Dorpat, Esthonia.)

[Plates 1 and 2.]

I.

Quantitative problems are, no doubt, of the greatest theoretical and practical interest in the study of the internal secretion of the sexual glands. We are explaining different physiological and clinical situations by changes in the quantity of internal secretion of the sexual glands or of other glands of internal secretion connected with the former. It suffices to mention normal puberty, menstruation and gravidity, eunuchoïdism and pubertas præcox. Quantitative problems were already discussed when the first steps were made in the study of the question of the site of the internally secretory function of the sexual glands (1). Bouin and Ancel (2) tried to cause by different experimental means a compensatory hypertrophy of the interstitial cells of the testicle; they extirpated the testicle in rabbits on one side and ligatured the vas deferens on the other side; they found a proliferation of the interstitial cells, whereas the number of the cells of Sertoli remained unchanged. Further, they extirpated the normal testicle of pigs with unilaterally retained testicle (3). They found in these experiments that the weight of the retained testicle was about twice as much as when the normal testicle was present. They found also in these cases a marked hypertrophy of the interstitial cells. Sand (4) confirmed these statements in his experiments on rabbits and guinea-pigs using his method of experimental cryptorchism. From all these experiments one could conclude that the hypertrophy of the interstitial cells takes place as a compensatory hypertrophy of the elements acting as an organ of internal secretion.

But there are some objections one can make against this conception. Ribbert (5) has shown that after unilateral castration the remaining testicle is greater than normally, and that there is a marked hypertrophy of the seminiferous part of the testicle. From experiments performed in our laboratory, we can state (6) that the hypertrophy of the remaining testicle is so marked that it weighs twice or thrice as much as a normal testicle of an animal of the same age. Sand showed that the remaining testicle can undergo the same hypertrophy even when the vas deferens is ligatured; this is due to the fact that, even after the vas deferens is ligatured, the spermatogenesis can proceed in a normal way, and the degeneration of the tubules begins only when the spermatogenesis is more or less completed. So one may object that, when the testicular mass is diminished, there is hypertrophy not only of the interstitial cells but also of the seminiferous part. This is why I said, two years ago (7), that the situation seemed to be more complicated than Bouin and Ancel supposed, basing their conclusions on ingenious experiments, performed about twenty years previously, when nobody could foresee the extraordinary development which the study of the internal secretion of the sexual glands has undergone in recent years.

We again took up the question of the hypertrophy of the interstitial cells in the testicle in connection with another problem of internal secretion of the sexual glands. We studied the question as to how the development of the sexual characters of mammals depends upon the quantity of secretion of the sexual glands present in the body. For this purpose we used a method consisting of making unilateral castration and of cutting away more or less from the second testicle ("partial castration") (8). We made a great many of these same experiments on guinea-pigs, and we were able to state—in accordance with Pézard (8A)—that even very small particles, representing 1 or even less than 1 per cent. of the normal testicular weight, are sufficient for a normal masculinisation of an animal (9). In some cases we observed that the development of the sexual characters was slower than normally; but we have experimental evidence that this phenomenon of retardation was caused, not by a simple quantitative deficiency in internal secretion, but by a slower development of the sectioned testicle (10).

In all these experiments we had theoretically to confront a very important complication, *i.e.*, the possibility of a compensatory hypertrophy of the elements, to which we ascribe the function of internal secretion in the testicle. And indeed in some cases where the particles were especially small, we found an extraordinary development of the interstitial tissue. The size and the number of the interstitial cells may sometimes be enormous. In such a small segment of the upper pole of the testicle, nourished by the arteria spermatica interna, the number of interstitial cells may no doubt attain, or even greatly surpass, the number of interstitial cells in two normal testicles together, although such a particle represents, as mentioned, not more but even less than 1 per cent. of the weight of two normal testicles.

I never saw such an enormous hypertrophy of interstitial cells as in these small particles in upper partial castration; only one such case of enormous hypertrophy of interstitial cells is to be found in the literature, in a paper recently published by Poll (11), who described the degenerating testicles of hybrids of birds. Anyone looking at a microscopical preparation of some of our cases, and comparing it with a normal testicle (Plate 1, fig. 1), would agree that the hypertrophy of the interstitial cells is here excessive—as in some tumours described in the pathology of the testicle in man.

In view of this hypertrophy one might object that even in our smallest particles there may have been a production of the sex specific secretion no smaller than the normal. It is true we have no definite evidence that the interstitial cells are the organ of internal secretion in the sexual glands; but there are so many facts showing that the interstitial cells have something to do with the internal secretion of these glands, that it is impossible to avoid this objection when we discuss the quantitative problems in the internal secretion of the sexual glands on the basis of experiments with partial castration. Whether or not the interstitial cells are really the organ of internal secretion in the testicle, the partial castration was, at any rate in some cases, counteracted by an hypertrophy of this organ.

We have some experimental evidence that this hypertrophy of interstitial cells is not a compensatory one, *i.e.*, that this hypertrophy is caused, not by an exaggerated function of these cells for the body as a whole, but by local conditions.

II.

The experimental evidence we have that the hypertrophy of the interstitial tissue is not a compensatory one, is of four different orders.

A. Going through all the cases where small particles of testicular substance were sufficient for a masculinisation, in different degrees, of guinea-pigs, we saw all transitions between a normal number of interstitial cells and a highly augmented number of the latter. But there seemed to be no constant relation between the number of interstitial cells and the degree of development of the sexual characters. We will give in another paper a full description of all our experiments with partial castration, considering them from the point of view of the problem of the site of the function of internal secretion in the testicle. Here only the following facts are of importance for us:—

(1) That there seems to be no constant relation between the number of interstitial cells and the degree of masculinisation, although there does not exist a case where masculinisation took place without fully developed interstitial cells being present in the testicular fragment.

(2) That a normal masculinisation is possible even when the number of

interstitial cells in a small testicular fragment is not very much augmented, so that the number of the interstitial cells is highly diminished in comparison with those in a normal testicle.

B. In the experiments with partial castration on guinea-pigs, mentioned above, we used in reality two different methods. In some of these experiments we left in the body, as previously said, a small segment of the upper pole of one testicle. In other experiments of this series we left a segment of the under pole of the testicle above the cauda epididymidis. In the latter we never saw the enormous hypertrophy of interstitial cells observed in some cases of "upper" partial castration, although in "under" partial castration a marked increase in the number of interstitial cells occurs. But the "under" testicular fragment degenerates, in general, so far as to become sclerotic, whereas the upper fragment can resist longer against sclerosation (11A). We explain this dissimilitude by a difference in the blood supply in the two methods. In the "under" partial castration the testicular fragment is supplied with blood by the arteria deferentialis, the artery of the vas deferens, which gives off branches from the under part of the testicle. These branches, as is known in human anatomy, have an anastomosis with the branches of the arteria spermatica interna supplying the upper half of the testicle. The art. def. is a small one in comparison with the art. sp. i., and we supposed that in our experiments the blood supply of an upper fragment was better than the blood supply of an under fragment. We found the plexus pampiniformis unchanged, so that it is very probable that a small upper testicular fragment received the same quantity of blood from the art. sperm. int. as the whole testicle. We think it right to conclude from these observations that the good blood supply explains, in a sufficient manner, the great development or the hypertrophy of the interstitial tissue in upper testicular fragments as related above.

C. Experimental evidence that the latter conclusion is true, and that the hypertrophy of the interstitial cells in the upper testicular fragment is caused by local conditions, is shown by the following observations. On six guinea-pigs of different ages the one testicle was cut into two fragments, both of which were left in the body; the upper one supplied by the art. sp. i., the under one supplied by the art. defer. (On the other testicle we made—for other experimental purposes—incisions going through about half or more of the testicle, but not touching the ductus epididymidis.) All these animals showed during four months of observation normal somatic sexual characters. A *résumé* of the six experiments we performed is given in the following Table:—

	No. of Protocol.	Duration of experiment.	Weight of animal.		Condition of upper fragment.	Condition of under fragment.
			At beginning.	At end.		
		days.	grm.	grm.		
I	*69*	123	230	430	Upper fragment grown together with under fragment. Tubules in full spermatogenesis; spermatozoa found in caput epididym. In the neighbourhood of the under fragment, the tubules are in the stage of desquamation, or in the juvenile stage. *Well developed interstitial cells in normal or slightly increased quantity.*	Some tubules with spermatozoa (?) and tubules with one stratum only. Fragment undergoing sclerosation. A few well-developed interstitial cells.
II	*70*	126	230	525	All seminiferous tubules in juvenile stage. *Interstitial cells in slightly increased quantity.*	No remains of fragment found.
III	*64*	128	280	580	Full spermatogenesis; spermatozoa. Some tubules with one stratum only. *Interstitial cells in markedly increased quantity.* Connective tissue grows inwards from level of incision.	Sclerosation.
IV	*76*	114	400	580	Seminiferous tubules with one stratum only. *Enormous hypertrophy of interstitial cells.*	Sclerosation. A few seminiferous tubules found, and a few well-developed interstitial cells.
V	*68*	123	290	510	Both fragments grown together. Sclerosation.	No testicular tissue recognised; only few interstitial cells (?)
VI	*75*	114	505	650	Sclerosation. Degenerating seminiferous tubules in upper and in under fragment.	

As we see from the weight of the animals used for these experiments, they were all at an age when the spermatogenesis in guinea-pigs has attained a very high degree, or when the production of spermatozoa begins; some of them were adult animals.

The result of these experiments is that in all the six cases there was, four months after the operation, a very marked degeneration of the under fragment of the operated testicle. This degeneration concerned both the seminiferous and the interstitial part of the testicular fragment, the latter being transformed more or less completely into connective tissue. Having observed a great number of testicles under different experimental conditions, I should like to mention here that there seem to be different forms of degeneration which the testicle may undergo; but I have not enough insight into this field of pathological anatomy to judge on this question.

Unlike the under fragment, the upper fragment was, in four cases, still resisting degeneration and sclerosation. No. 69 showed in the upper fragment, four months after the operation, tubules with spermatozoa which were even present in the caput epididymidis. Other tubules in this fragment were in the state of desquamation or in the juvenile stage. In No. 70 all tubules are in the juvenile stage, corresponding to that of an animal about three weeks old. In agreement with Benda (11B), we mentioned in another paper that it is in reality not justifiable to speak about a "degeneration" of the seminiferous tubules occurring after ligature or section of the vas deferens, transplantation, radiation, and so on. There is in reality only a process which leads up to a juvenile stage, a process which occurs in an indefinitely smaller measure also in the normal testicle. To understand that there is no other change than a return of seminiferous tubules *en masse* to a juvenile stage, it suffices to compare a preparation of No. 70 with one of a normal animal about three weeks old, as given in Plate 1, fig. 2. I do not think that this "backward development," to use a notion of Eugen Schulz (11C) is the only possible way of reaction of the seminiferous tubules in different experimental conditions, and I do not think that a seminiferous tubule which has returned to a juvenile stage will always have the same destiny or life-history as a juvenile tubule in a normal testicle; on the contrary, in our upper fragments and in other experimental cases we several times observed complete degeneration of such tubules. Evidently, the same experimental condition which may lead to backward development may also lead to complete degeneration of these juvenile tubules.

In No. 64 the upper fragment showed about the same condition as No. 69 Some tubules showed spermatozoa, others were in the juvenile stage. Beginning from the level of the incision, connective tissue grows inwards in the testicular tissue and a few weeks afterwards this fragment would surely have been in the same condition of sclerosation as an under fragment. We found this condition in No. 68, where no testicular tissue was to be recognised in the mass of connective tissue. A degeneration of the upper fragment took place in No. 75, but in a somewhat different way. Another case, No. 76, is of the greatest interest for us. It is the fourth of the experiments where the upper fragment was still present and in good condition as compared with the under fragment already wholly degenerated. In this case (Plate 2, fig. 3) the seminiferous tubules have only one stratum of cells; I am not able to say whether there are here only cells of Sertoli or some spermatogonia also; the first is the more probable. The interstitial tissue was in a state of hypertrophy, like that in some cases of "upper" castration. This one case, where we have an enormous number of interstitial cells in an upper fragment, although the second testicle is present in the body, is sufficient to decide the question whether the hypertrophy of interstitial tissue in some cases of upper partial castration is a compensatory one or not; this hypertrophy is not a compensatory one, but one caused by local conditions.

D. Further experimental evidence is given by the following observations: Instead of sectioning the testicle near the upper pole, as in the foregoing experiments, we sectioned the testicle near the under pole and cut away a very small fragment of the under pole, together with the cauda epididymidis. We made this operation on both sides. In principle, this is the same operation as that performed unilaterally in the experiments reported in C, but with the difference that, instead of having a *small* fragment supplied by the art. sp. i. on *one* side, the animals of this series had *big* fragments on *every* side supplied by the art. sp. i. If an hypertrophy of the interstitial cells occurs under these experimental conditions also, it cannot be compensatory, because the quantity of testicnlar mass is not diminished by the operation.

We made three identical experiments, a *résumé* of which is given in the Table on page 139.

As we see, there was in two cases a very marked hypertrophy of the interstitial tissue, especially in No. 72, illustrated by fig. 4. The hypertrophy is not so striking as in some small upper fragments. But one must take into consideration that, on examining a great number of testicles even under identical experimental conditions, as already mentioned, all transitions in the quantity of interstitial cells exist; even the two testicles of the same animal treated in the same manner may show very striking differences as concerns the interstitial tissue and the seminiferous tubules. On examining

	No. of Protocol.	Duration of experiment.	Weight of the animal		Condition of testicle.
			At beginning.	At end.	
		days.	grm.	grm.	
I	*73*	54	470	495*	Both testicles grown together. All seminiferous tubules with one stratum only. *Interstitial cells in a markedly increased quantity.* Sclerosation at the level where the incision was made.
II	*72*	109	516	670	Right testicle: All seminiferous tubules in the juvenile stage. Sclerosation beginning at the level of the incision. *Very markedly increased quantity of interstitial cells.* Left testicle: Tubules in full spermatogenesis with spermatozoa, tubules in the stage of desquamation and with one stratum only. Sclerosation as on the right. *Increased quantity of interstitial cells.*
III	*60*	125	130	420	Both testicles grown together. A great number of seminiferous tubules in full spermatogenesis; others in the stage of desquamation and with one stratum only; some tubules in the juvenile stage. *Quantity of interstitial cells not increased.*

* The animal died of illness, and was weighed the last time nine days before its death.

the two testicles of a normal animal, one will also sometimes observe very striking differences in weight and in the development of the seminiferous and interstitial apparatus. Great differences are also to be found between two animals of the same litter. This is why it is often impossible to have real control animals in experiments where conclusions should be based on weight or age relations.

On looking at fig. 4, one will see that there is a striking resemblance between the condition of this testicle and the testicle of a young animal (fig. 2). The condition of the seminiferous tubules, the condition of the interstitial cells, and the distribution of the latter embedded in a granular or homogeneous mass, all these remind one in a very striking manner of the testicle of a guinea-pig of about 2 or 3 weeks of age, when the testicle of this species has entered on its rapid development to spermatogenesis and puberty. This juvenile stage of the testicle, which one can observe under experimental conditions, is all the more interesting in that our animal No. 72 was at the time of the operation fully grown, weighing already more than 500 grm. There can be no more striking instance of the fact that a "backward development" of the testicle is possible under experimental conditions.

III.

After all, there can be no doubt that the hypertrophy of the interstitial cells, as observed under different experimental conditions, has nothing to do with the function of the testicle for the organism as a whole, but that this hypertrophy is caused only by local conditions in the testicle itself.

Kyrle (12), who studied the hypertrophy of the interstitial cells under different experimental conditions, has suggested that this hypertrophy has something to do with a regeneration process which the seminiferous tubules are undergoing. This point of view is in connection with another conception of the function of the interstitial cells, namely, that these cells represent a trophic organ for the seminiferous tubules. The latter conception plays a great *rôle* in the attacks, made in the last few months by different German authors (13), upon the theory of Bouin and Ancel (which was supported and further developed by Tandler, Steinach, Sand and myself) that the interstitial cells are an organ of internal secretion.

This is not the place to discuss the question whether our conception of the internally secretory function of the interstitial cells is right or not. Indeed this conception will not remain unaltered and it is impossible now to say how much of this theory will permanently stand in view of the further development of scientific research in our special field. And, it may be, the results of the experiments made in our laboratory and reported by myself in this communication will be interpreted by some as a withdrawal in some measure from the position I held and tried to strengthen in my book on the "puberty gland." But on the other hand I think that there are not yet sufficient data to attribute definitely to the interstitial cells a special local function in relation to the seminiferous tubules, although the possibility of such a function cannot be denied, even though the interstitial cells should play a *rôle* as an organ of internal secretion. It is necessary for us to emphasise this point since Stieve in a recent publication (13) has seriously misrepresented our views.

Summary.

In experiments with partial castration one may observe in small testicular fragments enormous hypertrophy of the interstitial tissue, the number and the size of the interstitial cells being very markedly augmented. This hypertrophy is not a compensatory one, as is shown by the following experimental evidence:—

A. Hypertrophy of the interstitial cells is not present in all cases of partial castration and very small testicular fragments with a not very much augmented relative number, and consequently with a highly diminished absolute number, of interstitial cells may suffice for a normal masculinisation.

B. On comparing testicular fragments supplied by blood in different ways (a fragment from the upper and a fragment from the under pole of the testicle) one finds that the fragment which seems to be better supplied by blood shows more pronounced tendency to hypertrophy of interstitial cells.

C. Enormous hypertrophy of interstitial cells may take place in an upper testicular fragment even when the other testicle is present in the body.

D. Marked hypertrophy of interstitial cells may take place when we transform both testicles into upper "fragments," by sectioning the testicle near the under pole and by so cutting away only a very small quantity of testicular mass.

In view of all these experiments it seems clear that hypertrophy of the interstitial cells, as observed in different experimental conditions, has nothing to do with the internally secretory function of the testicle in its relation to the organism as a whole. This hypertrophy is caused by local conditions, and is not brought about in response to general compensatory requirements.

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DESCRIPTION OF PLATES.

(The figures are drawn by Miss L. Lehbert, Dorpat.)

PLATE 1.

Fig. 1.—Two sections through the testicle of a normal animal aged about 4½ months (Prot. No. 27). On the left: interstitial cells in form of a triangle between tubules in full spermatogenesis; on the right: interstitial cells embedded in a granular mass in the neighbourhood of blood-vessels.

Fig. 2.—Two sections through the testicle of a normal animal about three weeks old (Prot. No. 32). The interstitial cells are rich in protoplasm, the nucleus is large. In the left half the interstitial cells are embedded in the granular mass.

PLATE 2.

Fig. 3.—Section through the upper testicular fragment of an animal subjected to the "complex" testicular section (Prot. No. 76). The hypertrophy of the interstitial cells is enormous. The seminiferous tubules with only one stratum of cells (cells of Sertoli).

Fig. 4.—Section through the testicle of an animal subjected to the operation described in D (p. 138). (Prot. No. 72). The seminiferous tubules are in the juvenile stage, although the animal was fully grown when operated on. The operation was performed about four months previously. The number of interstitial cells is very markedly increased.

All the testicles or testicular fragments were fixed in the solution of Helly (solution of Müller with 5 per cent. formol) and stained by eosin and hæmatoxylin. Only fig. 4 is made from a fragment fixed in Flemming and stained by Heidenhain's iron-alum hæmatoxylin. All the preparations were made by Dr. Wagner.

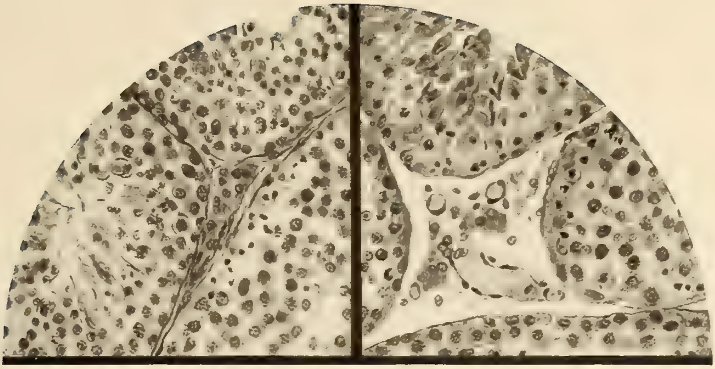

Fig. 1.

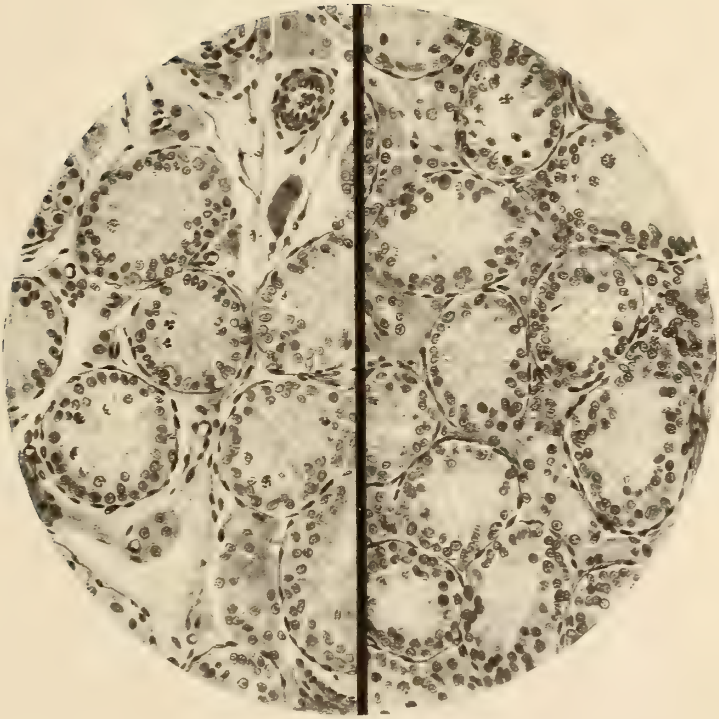

Fig. 2.

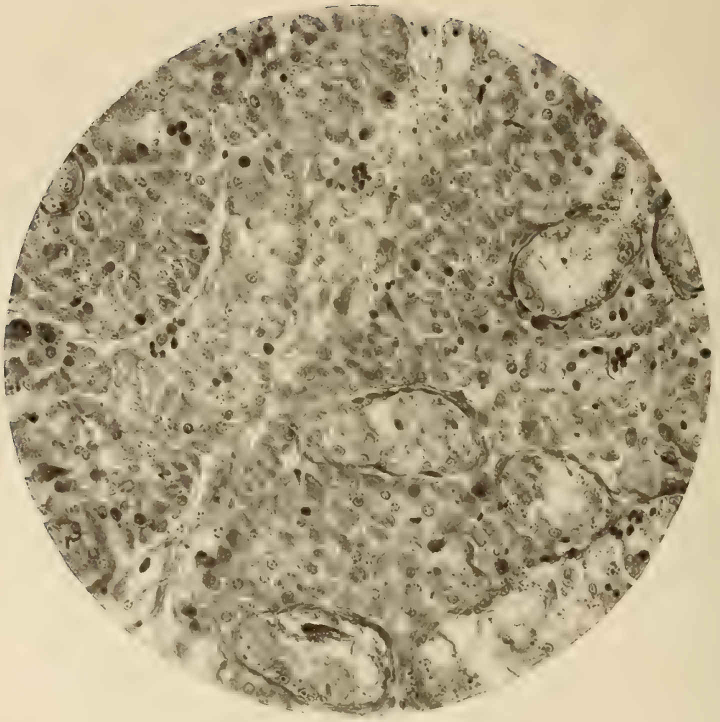

Fig. 3.

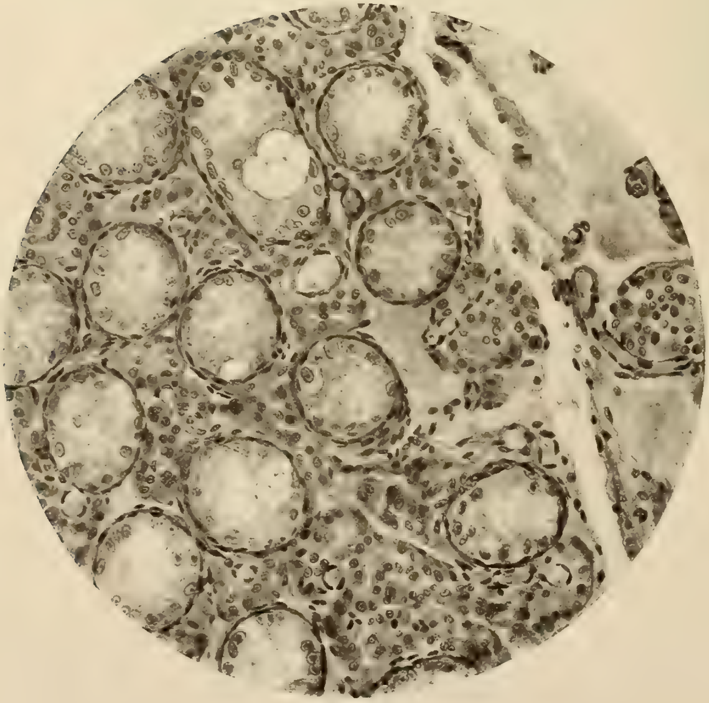

Fig. 4.

On the Irritability of the Fronds of Asplenium bulbiferum, *with Special Reference to Graviperception.*

By T. L. Prankerd, B.Sc., F.L.S. Lecturer in Botany, University College, Reading.

(Communicated by Prof. W. M. Bayliss, F.R.S. Received March 10,—Revised November 11, 1921.)

[Plate 3.]

1. *Introduction.*

Our knowledge of the phenomena of irritability in vascular plants is largely confined to Angiosperms. Very little work seems to have been done on Pteridophytes; and from his few observations on fern fronds, Darwin [(1), p. 509] even doubted if they possessed any power of response to gravity.

In a preliminary account (4) of the distribution of the statolith apparatus in plants, I mentioned that it was present in young fern fronds, and I have since found that this was partially known to Dehnecke (3). He referred to fern fronds as possessing non-assimilating chlorophyll grains resting on their physically lower cell walls; but he did not connect these grains with the perception of gravity, nor, so far as I am aware, have they ever been photographed or indeed noticed since.

An investigation, therefore, of the physiology and cytology of young fern fronds seemed advisable, in order to ascertain if, and how far, any connection could be traced between them. The common plant *Asplenium bulbiferum* proved a very suitable object for detailed study, though many of the observations described have been confirmed by work on other genera.

2. *The Growth and Movements of the Fronds.*

The plants used were grown in pots, generally under bell jars, in rooms each of which had only one window; thus subjecting them to one-sided illumination the direction of which was known. A few control observations were made on plants exposed to all-round illumination, and on others grown in the dark. The latter were very little used, because it was desired to study the behaviour of plants as far as possible under natural conditions. Many series of observations extended over weeks or even months, where prolonged darkness would have proved very deleterious, if not fatal.

The position of a frond under observation was determined by measuring as nearly as possible the angle made by the midrib with the horizontal. This was done by a very simple, but effective instrument consisting of a transparent protractor (A, fig. 1) mounted between two strips of glass (B) fitted upright into a slot in a wooden block (C). The two pieces of glass are held together, and keep the protractor in place, by an ordinary letter-clip (D) at the top. In order to bring the protractor on a level with the frond, it can be lowered by simply pressing it down from above, or raised by pushing it up from below, at the same time opening and holding down the clip to keep the supports in place. The latter are made of glass, so that the whole frond should be visible, and also to secure the easy adjustment of the 90° line of the protractor against the frame of the window—usually quicker than using a plumb line.

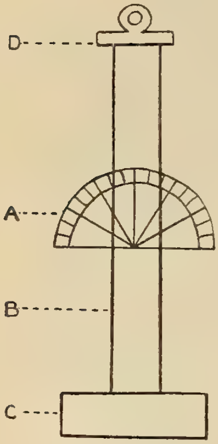

Fig. 1.—Instrument for measuring the angular height of a fern frond.

Three phases may conveniently be distinguished in the life-history of a fern frond; the first or infant phase when the apex of the frond is curled, the second or adolescent phase, from the time when the first leaflets appear beneath the apical coil, till the frond is quite uncurled, when the third or mature phase is reached. Growth cannot be measured as precisely in ferns as in angiospermic stems and roots, owing to the circinate vernation and the considerable individual variation in the process of uncurling. It frequently happens in the middle of the second phase, that the apex is raised very quickly (*i.e.*, in about 24 hours), so that the recorded height shows a greater increment than that due to growth in length. A fern frond, like the organs of angiosperms, shows a grand period of growth. When it first appears above the soil it grows slowly—0·5–1 mm. or so a day. This increases till a young frond 3–10 cm. in height, *i.e.*, in the second phase, may show a daily increment of 4–5 mm. In the third phase, growth slackens, and very gradually ceases altogether.

At least six types of movement are shown by fern fronds:—

(1) *Nutation.*—This is exhibited most strikingly in the second phase of growth, and the results I obtained for Asplenium are very similar to those recorded for *Nephrodium molle* by Darwin [(1), p. 257].

(2) *Rectipetality*, by which I mean the tendency in growth for the different parts of the mid-rib to be in the same straight line. This is sometimes apparently secured in the adolescent stage by an autotropic movement of part of the frond.

(3) *Sagging*, due to the weight of the developing pinnæ, is characteristic of the end of the adolescent and beginning of the mature stage, and gives the frond its final graceful form.

All these movements are slight; the first almost imperceptible to the unaided eye, the second merely occasional, and the third only occurs when growth is nearly over. They will, therefore, not concern us further, and we are left with the three most important movements, due to the following causes:—

(4) *Epinasty*, present throughout the first two periods, causes more or less striking curvatures towards the end of the adolescent phase. The tip of the frond, which may or may not be entirely uncurled, approaches the ab-axial side so that the upper part forms a loop, sometimes almost a complete circle, a position from which it eventually recovers by slight hyponasty.

(5) *Light* has a directive effect upon the frond which varies with the position of the latter, and does not seem to come into play till it is several centimetres in height, *i.e.*, in the middle of the first phase. Curvature is shown most strongly if the frond is placed abaxially to the light, when the apical part generally moves through 90° in the direction of the incident rays (see fig. 2, A).

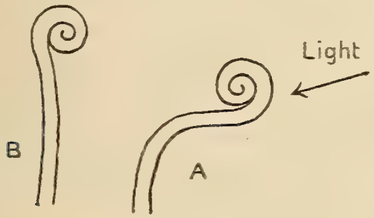

FIG. 2.

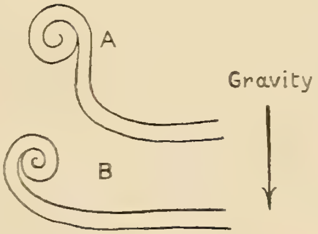

FIG. 3.

FIG. 2.—Explanation in text. (Imagine plane of paper vertical.)
FIG. 3.—Explanation in text. (Direction of light through plane of paper, which is imagined vertical.)

The effect is much less pronounced, and seems to come into play later, if the frond is placed so that the light strikes it laterally (*i.e.*, at right angles to the plane of the paper, fig. 2), when a slight positive movement takes place in the first period, and diaheliotropism, caused by a torsion of the rachis, is shown by the leaflets in the second. If, however, the frond is placed so that the incident rays strike the adaxial surface (fig. 2, B) little or no curvature takes place, but the frond grows approximately vertically well into the adolescent stage, since the effects of light and epinasty balance one another. The backward curve produced by the latter in the second period is considerably lessened.

(6) *Geotropism.*—Irritability to gravity exists from the inception of the frond till near the end of the second phase. It is best studied in fronds placed horizontally and adaxially to the incident light, when the organ will

curve through 90° to bring itself into the vertical. Placed horizontally but abaxially to the light, the frond will move into a vertical plane, at the same time curving towards the incident light. If fronds are placed horizontally, with their ab- or adaxial surfaces uppermost, the subsequent positions assumed are shown in fig. 3. In the former case (A) gravity and epinasty act together; in the latter (B) they are balanced against each other, though gravity seems to have the greater effect of the two, at least in the first phase, since an upward curvature is often produced, though never approaching 90°.

Although very little exact work has as yet been attempted, it is interesting to note that both the latent* and reaction† periods for gravity are probably considerably longer for ferns in general than angiosperms. In one experiment *Asplenium bulbiferum* was compared with Sidalsia sp. The frond of the one and the inflorescence axis of the other, neither unsevered from the parent plant, were placed horizontally in the open at about 27° C. In 4 hours a distinct geotropic curvature was visible in Sidalsia, and 1½ hours more proved sufficient to bring the axis back to the vertical, while no trace of curvature was to be seen in Asplenium. And while I have made few exact measurements‡ of the reaction time for ferns, it has never been as low as 5½ hours, except in Marsilia sp. at 18° C. The latent and reaction periods vary greatly with temperature and perhaps with the season of the year.

An attempt to express the relative effect of the last three factors and their magnitude in the different phases of the life-history of the fern frond is made in fig. 4, though the curves, with the exception of that of growth, are only approximate. In the former case, the ordinates are proportional to heights reached by the fronds on days represented by the abscissæ. The gravity curve rises with the growth of the frond, reaches a maximum in the adolescent stage, and ceases probably before the frond is entirely uncurled. It must, however, be understood that this, like other curves, attempts to express the average of many observations, and does not necessarily hold good exactly for any particular frond. The effect of light is represented by a curve which begins after that of gravity, rises in the second phase to the same height, and ceases probably soon after the beginning of the third phase, and therefore after that of gravity. Finally, epinasty§ starts with the inception of the frond, and is represented towards the end of the second phase higher than gravity or light, since its effect at this time is more pronounced than either, and seems to dominate the situation. It ceases at a period somewhere near the complete uncurling of the frond, but which varies greatly with the individual. Indeed, the last curve must be taken as even more approximate than either of the others, since epinasty is very variable, both in amplitude and the time of appearance and disappearance of its remarkable expression towards the end of the second phase.

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* The time elapsing between the first presentation of the stimulus and the beginning of the response.

† The time elapsing between the first presentation of the stimulus and the end of the reaction.

‡ The duration of these periods is now under investigation by my student, Miss F. M. O. Waight.

§ The subsequent relatively slight hyponasty is included here for convenience under the term epinasty.

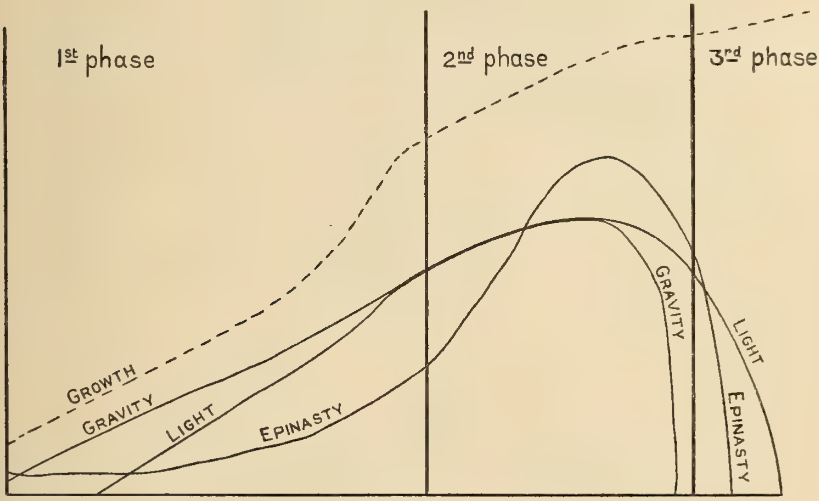

Fig. 4.—For explanation see text.

It is important for our purpose that the time at which geotropic irritability ceases should be ascertained as nearly as possible, but in actual practice this is very difficult, as the hyponastic curve is apt to simulate that due to geotropism, and the matter is complicated by the individual variability of the frond, which is most apparent in the very unstable adolescent stage. It seems at least certain that growth continues after the cessation of geotropic irritability, the frond being at this time only 70–80 per cent. of the length finally attained.

The fern frond is in many ways the biological equivalent of the angiospermic shoot, and, like it, will bring itself back into the vertical should it be displaced from this position. It accomplishes this by passing the vertical and swinging like a pendulum backwards and forwards till it remains upright in a manner similar to that well known for flowering plants, and originally, I believe, described by Darwin [(2), p. 508]. This takes place if the position of a frond is altered in the first, or in the earlier part of the second phase. Should, however, a frond be placed horizontally towards the end of this period, especially if the conditions are such that the reaction period is long, it will probably never reach the vertical, but remain at some angle, continuing its growth in the same straight line. In most cases when this occurs, there is a certain amount of oscillation before the frond becomes fixed in the oblique position.

Fig. 5 illustrates the life-history of an actual fern-frond moving under gravitational stimulus. The dotted line of growth is plotted as before, the other curve showing the rise of the frond, where the ordinates represent degrees. When the frond was only a centimetre or so in height, the pot was placed horizontally, and the frond slowly rose till it was at an angle of 60° with the horizontal. Like most fronds under these conditions, it never reached the vertical, probably in order to avoid the darkness due to the proximity of the soil. When the pot was placed upright, the frond attained the vertical position. Again placed horizontally, the frond rose to an angular height of 70° and then dropped. This was not due to the cause just explained, since the same thing has occurred in experiments when the pot was upright (see below). The frond is seen to oscillate for some time, and finally to take up a position at an angle of 45°, the recorded deviations from this being well within the limits of experimental error.

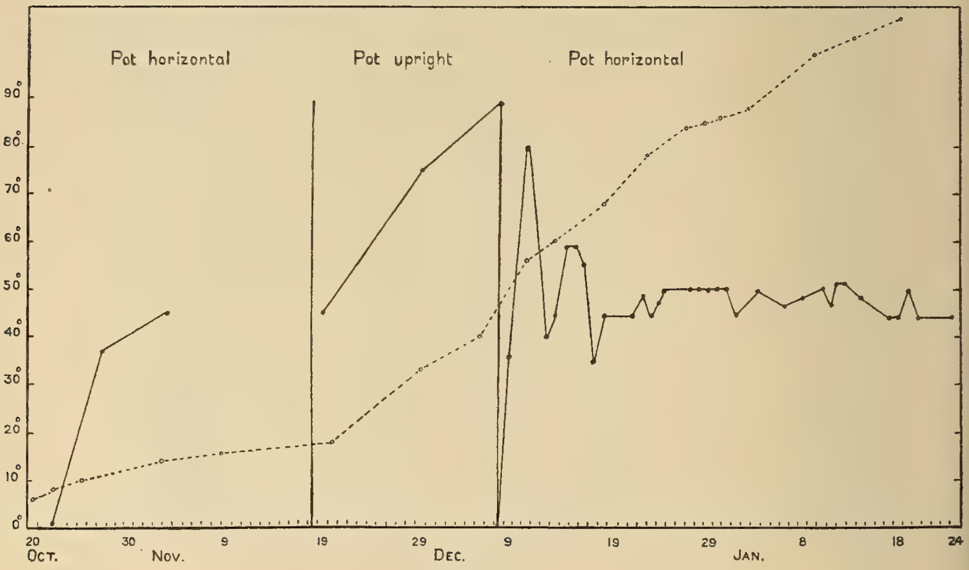

Fig. 5.

Text-fig. 6 is from a tracing made on glass of the actual position occupied by a fern frond on the recorded dates. A complete movement through 90° having been obtained by previously placing the pot horizontally, it was on

February 12 replaced in the upright position, making the frond therefore horizontal. It was slowly rising, till on the 18th it had reached an angular height of 45°. Round that position it oscillated, gradually uncurling, till this was complete on March 2. Soon after, the sagging effect due to the weight of the developing leaflets becomes apparent in the upper part of the frond. It will be seen that epinasty is not well marked in this particular case. As I read it, this record illustrates most of the movements described above, except that due to light, which was shown in the diaheliotropism of the leaflets not represented.

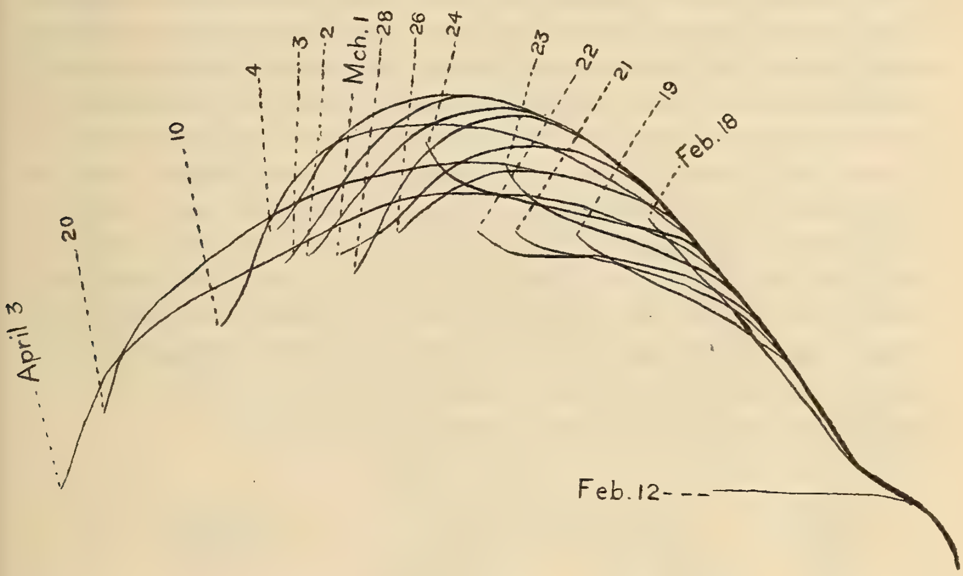

FIG. 6.

3. *The Statolith Apparatus of* Asplenium bulbiferum.

Most attention has been paid to the frond, though some stems have been examined. Starch does not seem to be very abundant in the rhizome of this plant, but was always found at the apex. In one case, in a stem which seemed dormant, very little was present, and it was quite unoriented. Statoliths are, however, always present beneath the rudiments of a developing frond, though some diversity is shown in the cytology of the statocyte. In one case the ground tissue was composed of cells with obvious statoliths, but possessing in addition starch grains round the nucleus, which latter was situated in the middle of the partial statocyte. In another case the nuclei were seen among the statoliths, though probably only loosely attached to them, if at all. This, however, was the only case in which any orientation of the nuclei in *A. bulbiferum* was ever observed.

The very youngest tissue—an actual meristem—never contains statoliths, which is scarcely possible, since little or no sap cavity is as yet developed in which they can lie loose; but, as the young frond develops, the chloroplasts about the bundle at the base become free, and this gradually extends outwards, till practically the whole green part of the ground tissue is composed of chloro-statenchyma (photo. 3). This well-characterised tissue (4) follows, so to speak, the growth of the petiole upwards, by being freshly formed as development proceeds to within a centimetre or so of the apex, and dying away behind as the xylem becomes lignified, and, later still, sclerenchyma is developed. Since more statenchyma is formed apically than dies away towards the base of the petiole, the amount gradually increases, till it probably reaches a maximum as the lower pairs of leaflets develop. It never seems to be found higher than the third or fourth pair, and dies away altogether before the end of the adolescent phase (fig. 7).

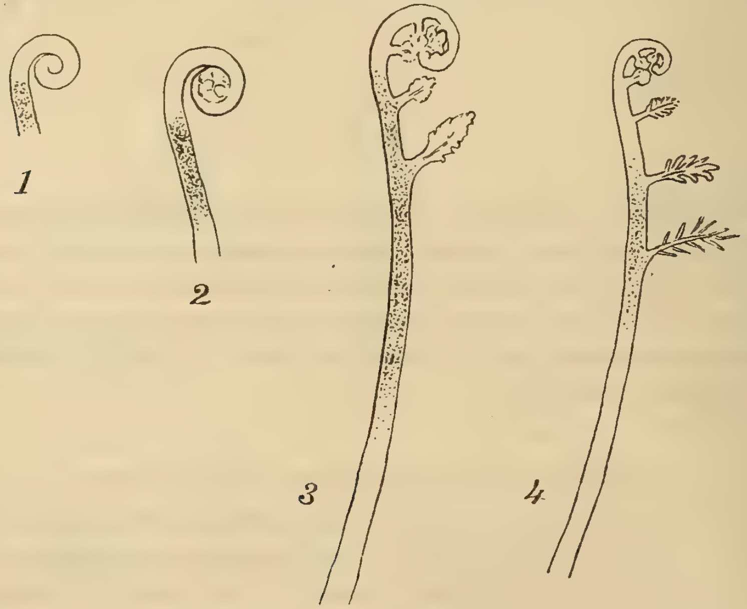

Fig. 7.—Fronds of *Asplenium bulbiferum* increasing in age from (1)–(4). Note that (4) is older morphologically than (3) though smaller. Dotted area indicates amount of statenchyma.

The statolith apparatus is very beautifully shown in the fronds of *Asplenium bulbiferum*. The statoliths are large, bright green bodies full of starch, and apparently indistinguishable from chloroplasts, except that they are completely free from the protoplasm and nucleus. Nearly every cell of the inner ground tissue (at the right time and place, see above) is a statocyte, and practically every chloroplast a statolith, so that we have a very complete, if not the highest, type of apparatus. In good transverse sections cut from fronds laid horizontally for an hour or two, the little heaps of chloro-statoliths may easily be seen with a simple lens lying on the walls which have been lowest. The statocytes are relatively large in cross-section, though shorter than usual in length, being only about twice as long as they are broad.

4. *Summary and Conclusion.*

From the foregoing facts, it will therefore be seen that the life-history of a fern frond of *Asplenium bulbiferum* falls naturally into three periods, characterised not only by external morphology, but by physiological response and cytological differentiation. This may be expressed in tabular form, thus:—

Stage.	Infant.	Adolescent.	Mature.
Frond	Curled	Uncurling	Uncurled.
Heliotropic irritability	Beginning	At a maximum	Ceasing early.
Geotropic irritability	Increasing	„ „	Absent.
Statocyte tissue	„	„ „	„

Darwin's failure to recognise apogeotropism for ferns is accounted for by the discovery of (1) the much greater reaction time; and (2) the comparatively early disappearance of geotropic irritability in these plants. In his experiment [(1), p. 509],* the older frond was probably losing its power of response to gravity, and the period of horizontality (46 hours) would not always be sufficient to induce curvature in the case of a frond "with the tip still inwardly curled." Darwin does not give the exact stage of development of this frond, nor the temperature of the experiment—points both of which have been shown greatly to influence the time of reaction.

Next in interest to the demonstration of the existence of both gravi-perceptive power and the possession of statoliths by fern fronds, comes their very close association. This has, of course, been noted for angiospermic shoots (2), but in fern fronds it is more striking. In the first place, a considerable part of the growth of the frond takes place after both its statoliths and its irritability to gravity have disappeared, but when it is *still able to respond to light;* and, again, these disappearances are, as far as can be ascertained, synchronous.

---

* I demonstrated apogeotropism in the identical species used by Darwin—*Nephrodium molle*—at Kew Gardens in 1916.

Graphically, this is shown in fig. 4, where a curve for the rise and fall of the statolith apparatus would practically correspond with that of response to gravity—certainly more nearly than with any other. The result of this work may therefore be held to support the view that the possession of statoliths is causally connected with graviperception in plants.

All acknowledgements will be made on the completion of my work on the cytology of the statolith apparatus in plants.

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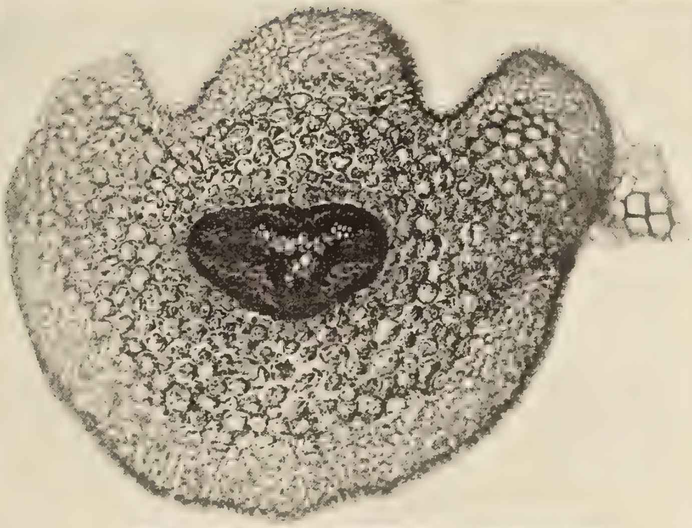
EXPLANATION OF PLATE.

Photomicrographs of transverse sections of a frond of *Asplenium bulbiferum* a little younger than (3) in text-fig. 7.

(1) is taken above the second pair of leaflets, and within the curl. The chloroplasts are scattered in the cells, and there is no trace of statoliths.

(2) cut just above the first pair of leaflets. The ground tissue is transitional between (1) and (3); statoliths are beginning to be formed.

(3) from below the first pair of leaflets—nearly all the cells of the ground tissue are statocytes.



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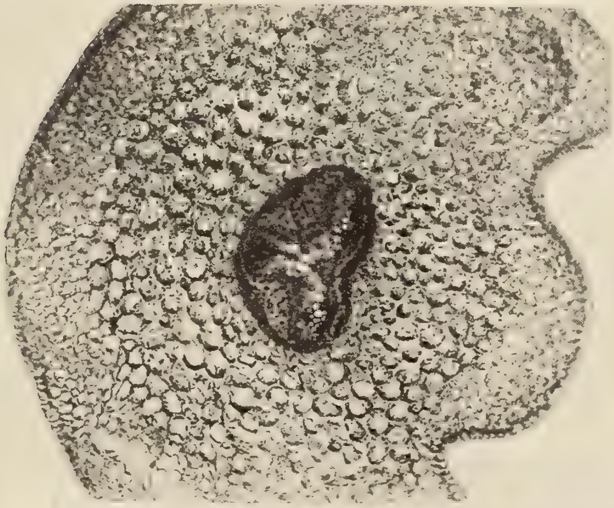

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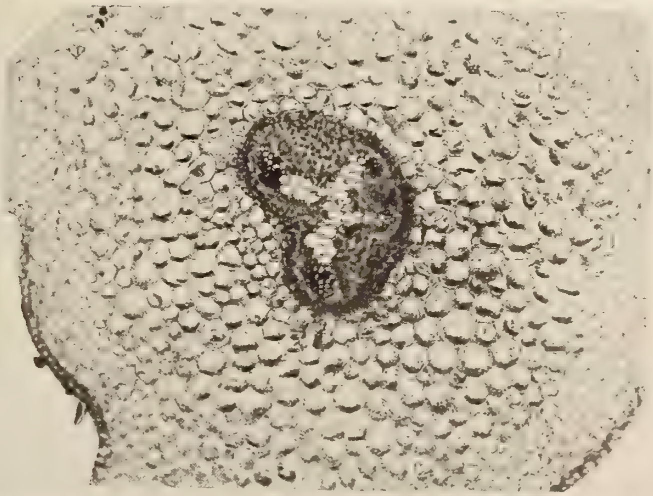

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The Dia-Heliotropic Attitude of Leaves as determined by Transmitted Nervous Excitation.

By Sir Jagadis Chunder Bose, F.R.S., Director, Bose Institute, Calcutta. Assisted by Satyendra Chandra Guha, M.Sc., Research Student, Bose Institute, Calcutta.

(Received May 25, 1921.)

The leaves of plants adjust themselves in various ways in relation to the incident light. The heliotropic fixed position is assumed by means of curvatures and torsions of the motor organ which may be the pulvinus, or the petiole acting as a diffuse pulvinoid. In some cases the motor organ alone is both perceptive and responsive; in others, the leaf blade does exert a directive action, the perceptive lamina and the motor organ being separated by an intervening distance. This directive action of the lamina has been found by Vochting in Malva verticillata, and by Haberlandt in Begonia discolor, and in several other plants. In connection with this it should be borne in mind that this characteristic does not preclude the possibility of the motor organ being directly affected by the stimulus. In a nerve-and-muscle preparation, the muscle is excited, not merely by indirect but also by direct stimulus. As regards the heliotropic adjustment of leaves, the stimulus of light acts, in the cases just mentioned, both directly and indirectly, the indirect stimulation being due to some transmitted effect from the perceptive lamina. We may regard the coarse adjustment to be brought about by direct, and the finer adjustment by indirect stimulation.

Certain leaves thus assume a heliotropic fixed position so that the blades are placed at right angles to the direction of light, the directive action being due to certain transmitted reaction, yet unknown. No explanation has, however, been forthcoming as regards the physiological reaction to which this movement must be due. Suggestions have been made that the dia-heliotropic position of leaves is of obvious advantage, since this position assures for the plant the maximum illumination. But such teleological considerations offer no explanation of the definite physiological reaction. It is, moreover, not true, as I shall show in the course of this paper, that there is anything inherent in the plant-irritability by which the surface of the leaf is constrained to place itself perpendicular to the incident light.

I have for many years been engaged in pursuing investigation on the subject, and have recently succeeded in discovering the fundamental reaction to which the directive movement is due. I shall be able to show that the particular attitude assumed by the leaves is brought about by transmitted "nervous impulse," which impinges on the motor organ, which is not simple but highly complex; that there are several distinct impulses which react on the corresponding effectors grouped in the motor organ.

For a full and satisfactory explanation of the phenomena, it will be necessary to deal briefly with the characteristics of the motor organ and the nervous impulse which actuates it. It will also be necessary to show that the physiological reactions of the "sensitive" and "ordinary" plants are essentially similar. As a type of the former I shall take *Mimosa pudica*, and for the latter, *Helianthus annuus*. I propose to deal with the subject in the following order:—

I.—General description of the dia-heliotropic phenomena.

II.—Characteristics of the motor organ:

1. Mechanical response due to differential excitability of the pulvinus of Mimosa and pulvinoid of Helianthus.
2. Response to stimulation of adaxial and abaxial halves of the motile organ.
3. The mechanism of heliotropic curvature.
4. The diurnal movement.
5. Torsional response to lateral stimulation.

III.—The nervous mechanism in plants:

6. Receptor, conductor, and effector.
7. Localisation of nervous tissue in plants.

IV.—The transmitted nervous impulse:

8. Definite innervation.
9. The directive action of propagated impulse in heliotropic leaf adjustment.

I.—General Description of the Dia-heliotropic Phenomena.

Before entering into the experimental investigation of the subject, it is desirable to describe the dia-heliotropic phenomena, as typically exemplified by Mimosa and Helianthus. A photograph of the former is reproduced in fig. 1, *a*, in which the plant placed in a box had been exposed to the northern sky and not to direct sunlight. It will be seen that the leaves which directly front the light have been raised, and so placed that the sub-petioles, with their leaflets, are at right angles to the strongest illumination. The side or lateral leaves have, on the other hand, undergone appropriate torsions—the plane of the leaflets being adjusted perpendicular to the light. It will be noticed that in executing this, the petioles to the right and the left have undergone opposite torsions.

After the assumption of this position, the pot containing the plant was turned round through 180°. This brought about a new adjustment in the course of twenty minutes, the plane of all the leaflets being once more at right angles to the light. The new adjustment necessitated a complete reversal of the former movements and torsions. Such perfect adjustment is brought about by bright light from the sky, and not so well by direct sunlight, for reasons which will be given later.

In fig. 1, *b*, is seen the heliotropic adjustment of the leaves of sunflower, grown near a wall, the plant being exposed to light from the western sky. The adjustment is essentially similar to that seen in Mimosa. The lateral leaves, 1 and 3, have undergone appropriate torsions—right-handed or left-handed—so that the leaf-blades placed themselves at right angles to the light. The leaf numbered 2 has been raised, placing its lamina perpendicular to the light. A contributing factor in this is the bending over of the stem, due to positive heliotropic curvature, which accentuated the rise of the leaf number 2. The same bending often causes an apparent fall of the leaf marked 4. When the stem is tied to a stake, the bending over of the stem is prevented; the leaf numbered 2 is then found raised by heliotropic action; but there is little or no fall of the opposite leaf.

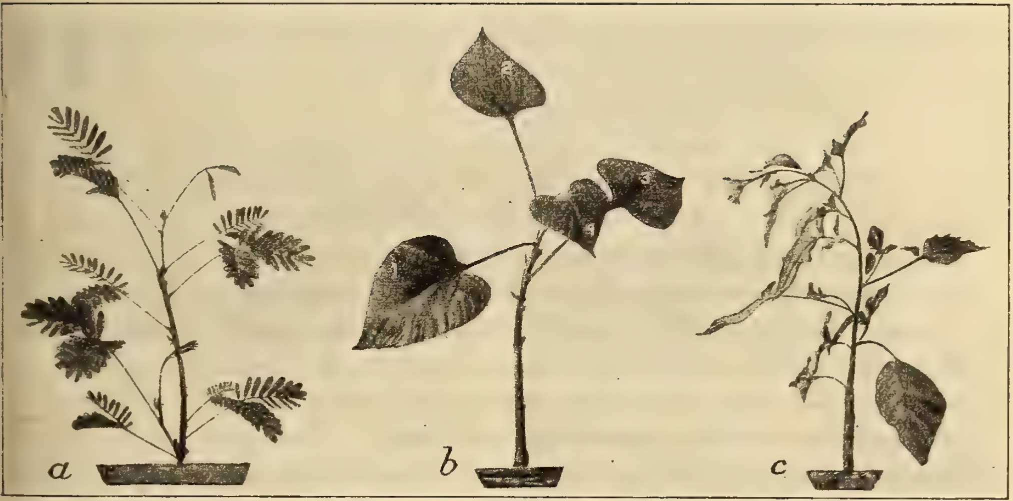

Fig. 1.—Dia-heliotropic adjustment of leaves: (*a*), in Mimosa; (*b*), in *Helianthus annuus*; and (*c*), in a different species of Helianthus. (From photographs.)

I reproduce (fig. 1, *c*) another photograph of the heliotropic curvature and adjustment of a different species of sunflower, which was grown in the open. In the morning the plant bent over to the east and all the leaves exhibited appropriate movements and torsions. In the afternoon the plant bent over to the west, all the previous adjustments and torsions being completely reversed. The plant continued to exhibit these alternate swings day after day till the movement ceased with age.

II.—CHARACTERISTICS OF THE MOTOR ORGAN.

I have shown elsewhere (2) that there is no essential difference between the response of "sensitive" and "ordinary" plants. I shall now show that all the characteristics of the response of the leaf of Mimosa are also found in the leaf of Helianthus. These will be specially demonstrated as regards normal response and recovery, the response of adaxial and abaxial halves of the organ to stimulus, the effect of direct and indirect stimulus in inducing heliotropic curvature, the daily periodic movements of the leaves, and the torsional response to lateral stimulation.

1. *Mechanical Response due to Differential Excitability of the Pulvinus of Mimosa and Petiole of Helianthus.*

In Mimosa, owing to differential excitability of the upper and lower halves of the pulvinus, a diffuse stimulus, such as that of an electric shock, causes a responsive fall from which there is a recovery on the cessation of stimulus. It has been thought that the upper half of the pulvinus is inexcitable. I have shown (3) that this is not the case, since local stimulation by light induces a contraction and resulting up-movement of the leaf. The upper part of the pulvinus is about eighty times less excitable than the lower half.

Experiment 1.—In Helianthus, the entire petiole acts as a motor organ, of which the upper half is relatively less excitable. Diffuse stimulation by electric shock induces a responsive fall, followed by a recovery on the cessation of stimulus. The response-records thus obtained are very similar to those obtained with the leaf of Mimosa. In Helianthus the reaction is relatively sluggish and the contraction is not so great as in Mimosa. The difference between the two responsive reactions is one of degree and not of kind.

2. *Response to Stimulation of Adaxial and Abaxial Halves of the Organ.*

As stated before, the upper half of the pulvinus of Mimosa responds to application of light by local contraction; the leaf is thus erected and the movement towards light may be described as positive heliotropism. The leaflets attached to the sub-petioles are thus made to face the light. Under strong and long continued sunlight the excitation is transmitted across the pulvinus, and causes at first a neutralisation, and finally a reversed or negative movement by the contraction of the more excitable lower half of the organ. This is the reason why the dia-heliotropic adjustment is less perfect under strong sunlight.

We obtain parallel reaction with Helianthus; here the petiole acts as an extended pulvinoid. Light applied from above causes an erectile movement; when applied below it causes a more energetic down movement. As the transverse conductivity of the petiole is feeble, the positive heliotropic response, induced by light acting from above, is rarely reversed into negative.

3. *The Mechanism of Heliotropic Curvature.*

A few words may now be said of the mechanics of curvature by which the stem of Helianthus bends towards light (fig. 1, *b*). All forms of stimuli, including that of light, induce a diminution of turgor and consequently contraction, and retardation of the rate of growth of the directly excited side. But this is not the only factor in bringing about the positive curvature. I have shown (4) that while the effect of direct stimulus at the proximal side of the stem induces diminution of turgor and contraction, its effect on the distal side, where it acts indirectly, is the very opposite, namely, an increase of turgor and expansion. The positive curvature is thus due to joint effects of direct and indirect stimulus at the two opposite sides. I have been able to demonstrate the induced increase of turgor at the distal side by experimenting with the stem of Mimosa. The stimulus of light is applied at a point directly opposite to the motile leaf, which by its movement indicates the change of turgor, the induced increase of turgor being indicated by an erection, and diminution of turgor by a fall of the leaf. Application of light at a point on one side of the stem was thus found to induce an increase of turgor at its diametrically opposite point.

Parallel experiments which I have recently carried out with Helianthus gave identical results. Arc light was continuously applied at a point opposite the indicating leaf; this induced an increase of turgor, as exhibited by a continuous erection of the leaf. We thus find that while direct stimulation induces a diminution of turgor at the proximal side, indirect stimulation causes an increase of turgor at the distal side. The positive heliotropic curvature is thus due to the joint effects of contraction of the proximal and expansion of the distal side.

4. *The Diurnal Movement.*

The daily periodic movements of the leaf Mimosa and of Helianthus exhibit a further similarity which is remarkable. I have shown elsewhere (5) that in plants sensitive to light the operative factors in the diurnal movement are :—

a. The variation of geotropic action with changing temperature. A rise of temperature is found to inhibit the geotropic action ; a fall of temperature accentuates it. In consequence of this the leaf, subject to geotropic action, undergoes a periodic up-and-down movement ; the maximum fall of the leaf takes place at thermal noon, which is about 2 P.M., the maximum rise is at thermal dawn, about 6 A.M.

b. The action of light is, generally speaking, antagonistic to that of temperature. In the forenoon, rise of temperature causes a fall of the leaf, but continuous light acting from above tends to raise it. The rapid diminution of light towards evening acts virtually like a stimulus, causing an abrupt fall of the leaf.

The diurnal movements of Mimosa and Helianthus exhibit four phases which are very similar :—

(1) The leaf, owing to fall of temperature, erects itself from 2 to 5.30 P.M., or thereabouts.

(2) After 6 P.M. there is a rapid diminution of light, and the leaf undergoes a sudden fall, which continues till about 9 P.M.

(3) After 9 P.M. the leaf begins to erect itself with the fall of temperature, the maximum erection being attained at thermal dawn, which is at 6 A.M., approximately.

(4) In the forenoon the leaf is acted on by two antagonistic reactions, the effects of rising temperature and of increasing light, the effect of rise of temperature being predominant. The leaf thus continues to fall till thermal noon, which is about 2 P.M.

5. *Torsional Response to Lateral Stimulus.*

I shall now refer to a very important type of responsive movement induced by lateral stimulus. A stimulus is called lateral, when it acts either on the right or the left flank of a dorsiventral organ. I shall presently show that a dorsiventral organ responds to a lateral stimulus by torsion. That this effect is universal will be demonstrated by experiments on the "sensitive" Mimosa, and the "ordinary" plant Helianthus. In order to eliminate the effect of the weight of the leaf, and also for obtaining record of pure torsion, the petiole is enclosed in a hooked support of glass, with a smooth internal surface (fig. 2). Friction and the effect of weight are thus practically eliminated ; the circular support prevents any up or down movement, yet allows freedom for torsional response. I have recently employed another device which is even more perfect. Instead of the tubular support, the petiole is slightly stretched in a horizontal direction by an attached thin elastic string of indiarubber tied to a rod. The up or down movement is thus prevented, whereas the string offers but feeble resistance to torsion. The torsion is magnified by an L-shaped piece of aluminium wire appropriately tied to the petiole, so that the long arm is at right angles to the petiole. The end of the arm is attached by a silk thread to the short arm of a recording lever, R; there is thus a compound magnification of the torsional movement, a right-handed torsion producing an up-curve, and a left-handed torsion a down-curve. The oscillating recorder gives successive dots at definite intervals of time, which may be varied, according to requirements, from twenty to sixty seconds. Time relations of response may thus be obtained from the dotted record.

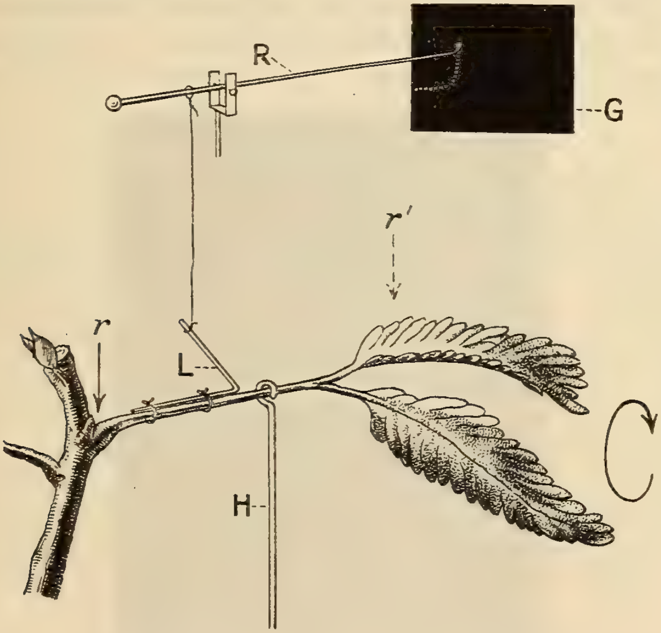

Fig. 2.—Diagrammatic representation for obtaining record of torsional response: H, hooked glass rod to secure pure torsional effect; L, bent piece of metal for magnification of torsional movement; R, recording lever; G, oscillating smoked glass plate. Direct stimulation of the right flank, *r*, or indirect stimulation of the right sub-petiole, *r′*, induces right-handed torsion.

Fig. 2 gives a representation of the experimental method for studying the torsional response of various "sensitive" and ordinary leaves. Diverse stimuli are applied at one flank of the organ, which is the junction of the unequally excitable upper and lower halves of the pulvinus or petiole. We shall presently find that the responsive reaction in all dorsiventral organs obeys a definite law in regard to the relation between the direction of incident stimulus and the resulting torsion. The torsion induced is either right-handed or left-handed, clockwise or anti-clockwise. In describing the direction of torsion, the position of the observer in regard to the plant must be definite; he should stand in front of the responding leaf and look at the central stem. When the right flank of the pulvinus or the petiole is struck by a horizontal beam of light *r*, coming from the right, the induced torsion is right-handed. Light acting on the left flank induces a torsion which is in the opposite or left-handed direction. On the cessation of stimulus the leaf recovers its normal position (fig. 3).

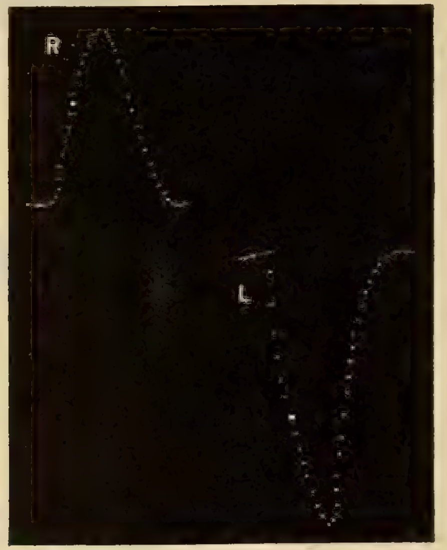

Fig. 3.—Record of torsional response of pulvinus of Mimosa by lateral light. Stimulation of the right flank induced right-handed torsion, R, represented by up-curve: stimulation of the left flank induced left-handed torsion, L. The two thick dots represent the duration of stimulus. Successive dots are at intervals of 20 seconds.

The response described above takes place when the pulvinus or the petiole is exposed to lateral light, the leaflets of the lamina being completely shielded from it. The differentially excitable organ thus undergoes a twist, in consequence of which the less excitable upper half of the pulvinus is made to face the stimulus. The leaflets or the lamina attached to the petiole are thus carried passively, like so many flags, to face the hypothetical source of light. It is obvious that the response is brought about by a definite physiological reaction and not for the utilitarian purpose of securing maximum illumination of the leaflets or the lamina. Teleological considerations, often adduced, offer no real explanation of the phenomena; such arguments are, moreover, highly misleading, for similar responsive torsion is induced, not merely by light, but by modes of stimulation so diverse as electrical, thermal, geotropic, and chemical.

Response of all anisotropic organs to lateral stimulus is included in the following generalisation:—

An anisotropic organ, when laterally excited by any stimulus, undergoes torsion by which the less excitable side is made to face the stimulus.

In a dorsiventral organ, it is the upper side which is, generally speaking, the less excitable. Hence the above generalisation may be expressed in the following simpler terms: Lateral stimulation of a dorsiventral organ induces a torsion which is right-handed, when the right flank is stimulated. Left-handed torsion is induced by the stimulation of the left flank.

Torsional response of petiole of Helianthus.—The above generalisation finds independent support from the response of the petiole of Helianthus to various stimuli applied laterally.

Experiment 2.—Two fine pins are thrust about 1 cm. apart on the right flank of the petiole of Helianthus, to serve as electrodes for application of induction shocks from a secondary coil; a similar pair of electrodes are attached to the left flank. On application of a feeble tetanising shock to the right flank, the petiole exhibited a right-handed torsion; stimulation of the left flank induced a left-handed torsion. Electric stimulation quickly stirs up the internal tissues, hence the latent period is short, and the responsive reaction is rapid (fig. 4, *a*). I next took a different specimen, and applied the stimulus of light to the right and the left flanks alternately. This gave rise to right- and left-handed torsions as under electric stimulus, the only difference being in the slower reaction and prolonged latent period (which was 15 minutes) (fig. 4, *b*). It must be remembered that in the case of light the excitation is gradually transmitted from the outer surface to the inner tissues. As regards the direct action of light, the results given above show that the responsive reactions of sensitive and ordinary plants are not different, but essentially similar. With reference to the heliotropic adjustment of leaves, we found that, when light strikes symmetrically in front, the leaf bends towards it. The growing stem itself is excitable, and its induced curvature is a contributory factor in placing the surface of the lamina at right angles to the light. Leaves struck laterally by light undergo torsion which is definite, being determined by the direction of the incident light. The torsion thus induced places the leaflets of the lamina at right angles to the light. These effects are produced, as stated before, when the responding pulvinus or petiole are directly exposed to light, the leaflets and lamina being protected from it.

The heliotropic adjustment of leaves often takes place, as we have seen, when the motor organ is in the shade, or is artificially kept so. There must, therefore, be transmitted impulses by which the distant motor apparatus is so actuated that the leaflets or the lamina are placed at right angles to the light. The transmitted impulse, if single or diffuse, cannot evidently exert the necessary directive action. I shall presently show that the transmitted impulse is of a nervous character, that the impulses are more than one, and distinct from each other, and that they travel by different channels from the lamina which perceives light to the distant motor region where movement is effected in response to transmitted excitation.

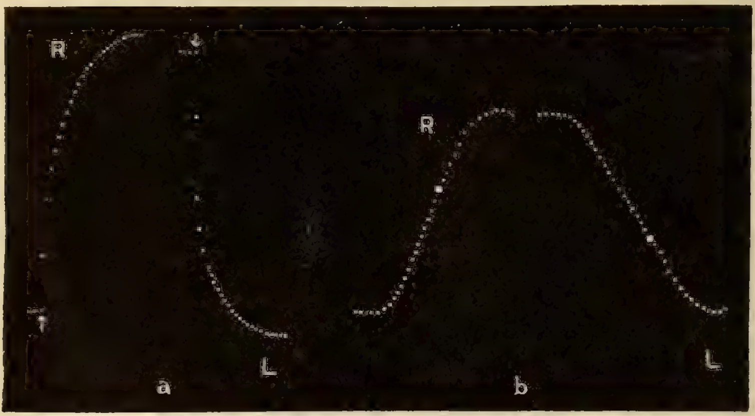

Fig. 4.—Torsional response of petiole of Helianthus in response to (a), electric stimulus, and (b), to stimulus of light. R and L are the opposite responses, due to stimulation of the right and left flanks. Successive dots are at intervals of 20 seconds. The prolonged latent period under light is not shown in the record. The portion of record exhibiting recovery is also omitted.

The Complex Character of the Motor Organ.—As regards the motor organ itself, I have stated that it is not simple but very complex. This will be understood from the following experiments on the pulvinus of Mimosa. Let us imagine the pulvinus to be diagonally divided into four quadrants. When the upper quadrant is subjected to light acting from above the responsive movement of the leaf is upwards; stimulation of the lower quadrant by light acting from below induces a down movement. The stimulation of the right quadrant (right flank) by a horizontal beam of light induces, as we have seen, a right-handed torsion; the left quadrant responds by a left-handed torsion. The four quadrants may, therefore, be regarded as four independent effectors, the resulting movement being determined by their combined effects. We may distinguish these four effectors as the upper, the lower, the right and the left effectors. We shall presently find that these different effectors are set in action not merely by direct stimulation, but by the transmitted impulses from a distance, along definite conducting strands by which the different effectors are definitely innervated.

III. The Nervous Mechanism in Plants.

I shall now describe the "nervous" mechanism by which stimulus received at the receptive end gives rise to an excitatory impulse, which is conducted along certain definite channels. It is necessary here to justify the use of the terms nervous tissue and nervous impulse in regard to plants, since the idea has long been prevalent that there is nothing in plants which corresponds to the nervous impulse in animals. The transmitted effect of stimulus in Mimosa was thus regarded not as a propagated excitation but merely a hydro-dynamical disturbance. I have shown, however (7), that the transmitted impulse is of an excitatory character, that it may be blocked by various physiological blocks, and that, like the nervous impulse in animals, the velocity of transmission is enhanced with a rise, and depressed or even arrested by a fall of temperature.

6. *Receptor, Conductor, and Effector.*

The nervous system of plants must be regarded as of a comparatively simple type. In speaking of the evolution of the nervous system, Parker points out that the contractile tissue or muscle appeared first as an independent effector, and that the nerve developed secondarily in conjunction with such muscles as a means of quickly setting them in action; that a receptor or sense organ alone would be of no service to an organism, neither would nerve or nerve centres alone; whereas a muscle cell or effector is of use if it can be stimulated directly (1).

In plants we find clear indications of these different stages. Thus in the leaf of *Erythrina indica*, and in the terminal leaflet of *Desmodium gyrans*, the pulvinus is the independent effector, the connecting nerve link being absent or functionally ineffective; heliotropic movement thus takes place when the pulvinus is directly stimulated, illumination of lamina having no effect. In Mimosa and in Helianthus, on the other hand, the intermediate nerve network has, as we shall find, become effective, the leaflets or the lamina serving as receptive organs. Haberlandt (9) has shown that in many cases the epidermal cells of leaves are of a lenticular shape, for increasing the perception of light. He rightly observes that "in zoological nomenclature, organs concerned with the perception of external stimuli have always been known as sense organs, even among lower animals and in other cases in which it is doubtful if the organs in question are responsible for sensation in the psychological sense. It is, therefore, not only permissible, but necessary in the interest of consistency to apply the term sense organ to the analogous structure in plants."

With regard to nerve and nervous impulse, I quote the following from Bayliss (1), italicising the important passages:—

"We find the presence of nerve at a very early stage of evolution. . . . The effect of anything happening at one end of such a thread is conveyed with great rapidity to the other end of the nerve, wherever it may be. *Nerve fibres have no other function than that of carrying excitation.* When set into activity by some influence, the disturbances set up disappear spontaneously after a very short time if the stimulus ceases to act. . . . *It is usual to speak of a 'propagated disturbance' passing along the nerve, or sometimes a 'nervous impulse.'* The most sensitive apparatus has been able to detect with certainty one kind of change accompanying the passage of the propagated disturbance, namely, an electrical effect."

All the characteristics of nerve described above are also found in the conducting tissues of plants. As regards the velocity of transmission of impulse, it is not so high as in higher, but not so slow as in lower animals. Thus in the frog's nerve the velocity is about 32 metres per second; in Eledone it is, however, as low as 1 mm. per second. The velocity in Mimosa is about 30 mm. per second. Though the propagated disturbance causes no visible change, yet the nervous impulse in plant, as in animal, may be detected by definite electric change of galvanometric negativity; the disturbance set up disappears spontaneously on the cessation of stimulus. If the electric contact be made only at one point of the plant nerve, the other being at a distant indifferent region, the electric response is monophasic. But if the contacts are made at two points of the nerve, the proximal is the first to become galvanometrically negative; the propagated disturbance then reaches the distal point with concomitant negativity of that point. We thus obtain the characteristic diphasic response of the nerve (see below).

Since the nervous reactions in animals and plants are so essentially similar, delay in full recognition of this fact will undoubtedly retard the advance of science. I shall in the present paper demonstrate certain striking effects in plants, which at first sight would no doubt appear as very astonishing, but which in reality result from nervous reaction, usually regarded as the special characteristic of the animal. I shall be able to show that in the plant a definite nervous link exists between the receptor and the effector, and that there is a well-developed system of innervation, by which the "attitude" of the plant-organ becomes adjusted to the incident stimulus.

7. *Localisation of Nervous Tissues in Plants.*

I have in my previous works shown that in Mimosa stimulus gives rise to an excitatory impulse, which is transmitted with a definite velocity, that this impulse has all the characteristics of the nervous impulse in animals. The most important problem in connection with this subject is the localisation of the conducting or nervous tissues. I succeeded in isolating a length of such a tissue in ferns and was able to obtain with it many results which are regarded as characteristic of nervous tissue in animals. In Mimosa, however, it is impossible to isolate the nervous tissues without injury, and I have for many years been confronted with the problem of localising *in situ* the particular tissue which serves as the conductor of excitation. I have recently been successful in my efforts, the method employed being that of the electric probe (6), by which I was able to localise the geotropic sense organ in plants.

Limitation of space enables me only to give the essential details of the method of localisation of nervous tissues and some typical results. A fuller account will be given in the forthcoming number of the 'Transactions' of my Institute.

The principle of the method will be understood if we take the somewhat analogous case of a cable along which electric messages are being transmitted. The conducting strand is here embedded in a non-conducting sheath. We can localise the embedded conductor and pick up the transmitted message by gradually thrusting in the electric probe, which is insulated except at the extreme tip. A galvanometer included in the circuit of the probe will begin to pick up messages that are being transmitted from the moment of contact of the tip of the probe with the conducting strand. The depth of insertion for contact can be read on a suitable scale and the position of the conductor may thus be determined.

We may similarly localise the exact position of the conducting nerve embedded in the petiole of Mimosa (fig. 5). Excitation of the sub-petiole will give rise to an excitatory impulse which travels in a centrifugal direction towards the stem. This excitatory impulse is of galvanometric negativity. The conducting nerve will be most intensely excited by the transmitted impulse, and the induced electrical change of this particular tissue will be maximum. Excitation will no doubt be irradiated to the adjoining tissue, but this will undergo a rapid diminution in radial directions outwards. If the stimulus be moderate or feeble the irradiation will be slight.

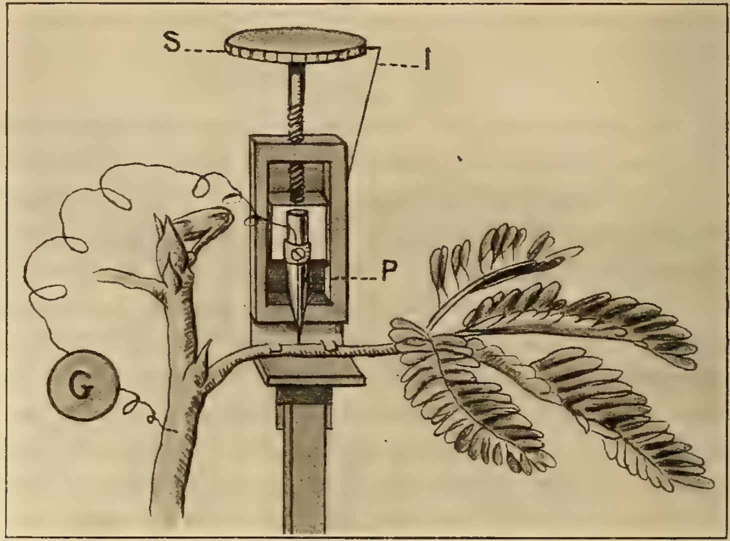

Fig. 5.—The Electric Probe for localisation of nervous tissue in plants. P, the probe in circuit with the galvanometer, G; S, the screw head, by the rotation of which the probe enters the petiole in successive steps; I, index by which the depth of intrusion may be determined.

The experimental procedure is as follows:—The probe is intruded perpendicularly along the diameter of the petiole. The intrusion of the probe is by steps, say of 0·05 or 0·1 mm. at a time. The slight wound produced by the insertion of the tip of the probe causes an excitation, which subsides completely in the course of about fifteen minutes. The sub-petioles are now stimulated by suitable stimuli, which may be chemical, thermal, mechanical or electric. The excitatory impulse is propagated preferentially along certain conducting channels in the petiole. The results to be described were obtained with all the different modes of stimulation. The electric mode of stimulation has the advantage that it can be maintained constant or varied in a graduated manner. Special precautions are taken that there should be no disturbance caused by leakage of the stimulating current; this is verified by the fact that reversal of primary current which actuates the secondary coil causes no change in the electric response; the excitatory electric change in different layers of tissue is, moreover, definitely related to the character of the tissue.

I shall anticipate results by describing the characteristic effects. The excitatory electric change detectable in different layers as the probe passes from the epidermis to the central pith is found to rise suddenly to a maximum in the phloem portion of the fibro-vascular bundle; the xylem shows little or no transmitted excitation. Hence we arrive at the conclusion that it is the phloem which functions as the nerve of the plant. The characteristic electric maximum was not found in experiments where the probe missed the phloem; greater experience now enables me so to direct the passage of the probe as not to miss the nerve tract.

In the diagram of the transverse section of the petiole of Mimosa usually given in text-books there is in each bundle a single phloem strand outside the xylem. I was, therefore, considerably puzzled by the fact that in traversing the bundle I obtained two electric maxima, one before reaching the xylem, and the second after passing it. In order to determine the cause of this anomaly I made transverse sections of the petiole of Mimosa. Differential staining clearly brought out the fact that the phloem strand is not single but double, one above and the other below the xylem. The second electric maximum coincided with the inner phloem.

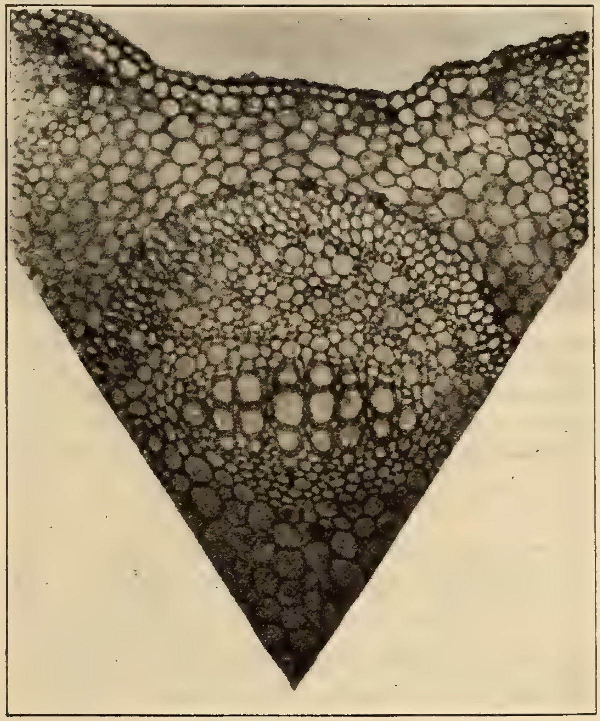

FIG. 6.—Micro-photograph showing a quadrant of the petiole and the fibro-vascular bundle. The tissues seen in the section are: the epidermis, the cortex, the bundle sheath, the first phloem, the xylem, the second phloem, and the central pith.

It may be stated here that in petioles provided with four sub-petioles there are four distinct bundles with four nerve trunks. But in specimens with two sub-petioles we only find two bundles, corresponding to the two sub-petioles. Two sub-petioles are found, generally speaking, in younger specimens. The micro-photograph (fig. 6) shows one of the bundles.

Experiment 3.—Electrical excitation in different layers: I shall now give detailed results of localisation of the conducting tissue. The probe enters the epidermis and is pushed in by steps of, say, 0·05 mm.; it passes in succession the cortex, C, the outer phloem, P, the xylem, X, the inner phloem, P′, and the central pith, O. The thickness of the different layers is modified by age of the specimen. In the records given below (fig. 7) the electric response of the epidermis = +12 divisions of the galvanometer. I have shown elsewhere (8) that the epidermis, which protoplasmically is more or less dead, gives either a zero or a positive, in contradistinction to the normal negative response of living tissues. The probe at a depth of 0·1 mm. encountered the cortex and the response there was −17 divisions. The phloem extended through 0·15 mm., the average depth being 0·2 mm. The response in this region underwent a sudden enhancement, as seen in the three responses −61, −65 and −40 divisions. The xylem which was at a depth of 0·3 mm. showed no response,

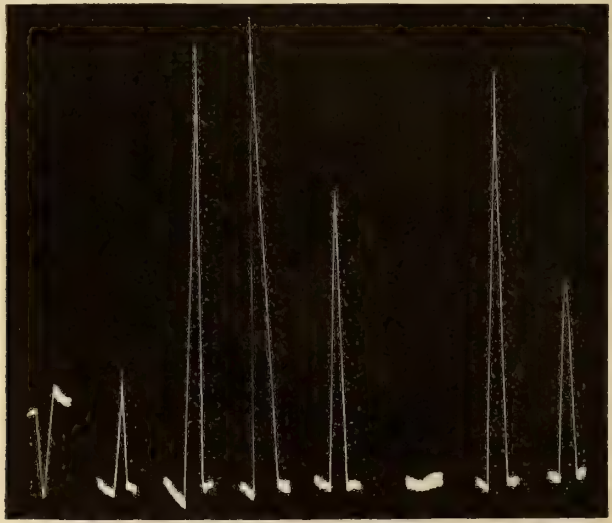

Fig. 7.—Galvanometric record of transmitted excitation in different layers of the petiole: the first is the positive response of the epidermis, the second is the feeble negative response of the cortex, the third, fourth, and the fifth are the enhanced responses in the first phloem, the sixth shows absence of excitation in the xylem, the seventh is the enhanced response in the second phloem, the eighth is the diminished response in the pith.

proving that it was a non-conductor; when the probe reached a depth of 0·35 mm. it encountered the second phloem, where the response underwent a second enhancement of −56 divisions. The probe reached the border of the pith at a depth of 0·4 mm. and the response underwent a diminution to −26 divisions. In cases where the incident stimulus on the sub-petiole is feeble the irradiation effects are greatly diminished; the excitatory transmission is then found only in the phloem. I give below a summary of results obtained with ten different specimens:—

Table I.—Showing Intensity of Transmitted Excitation in Different Layers in Ten Different Specimens.

Different layers.	Transmitted excitation.										
	I.	II.	III.	IV.	V.	VI.	VII.	VIII.	IX.	X.	Mean.
Epidermis...	+1	0	0	0	0	0	0	+4	0	0	+0·5
Cortex	−2	−3	−50	0	0	0	0	0	0	0	−5·5
Phloem	−30	−30	−100	−30	−36	−44	−33	−18	−20	−24	−36·5
Xylem	−8	−9	0	0	0	−10	0	−4	−8	−8	−4·7
Phloem	−30	−30	−84	−10	−36	−20	−12	−18	−20	−16	−26·8
Pith	0	−6	−29	0	0	−7	0	0	0	0	−4·2

It will be seen that in all cases the phloem is invariably found to be the best channel for conduction of excitation. The following curve (fig. 8),

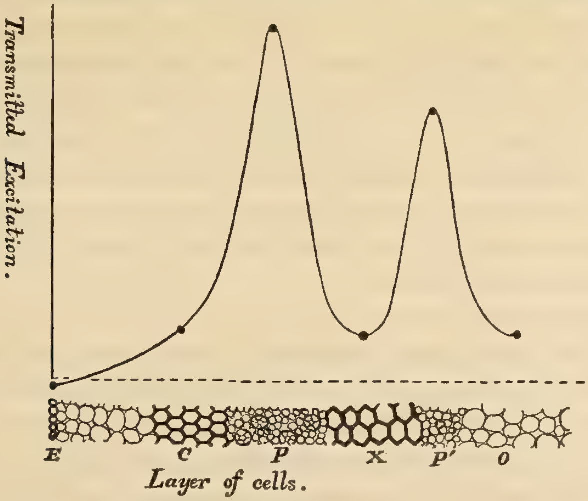

Fig. 8.—Curve showing the different intensities of transmitted excitation in different layers: E, epidermis; C, cortex; P, first phloem; X, xylem; P′, second phloem; O, pith.

plotted from the mean values given in Table I, illustrates this in a striking manner.

IV. THE TRANSMITTED NERVOUS IMPULSE.

In certain experiments with petioles having four bundles, I allowed the probe to pass vertically through the petiole when it encountered the upper and lower bundles. I thus obtained maximum transmitted excitations in the phloems of the upper fibro-vascular bundle, and a similar maximum in the phloems of the lower bundle, the intervening layers of tissue being practically non-conducting. From this it follows that excitatory impulse is propagated along definite channels through the length of the petiole.

8. *Definite Innervation.*

We shall now follow the nervous strand from the perceptive lamina to the motor organ. In Mimosa, the leaflets attached to the sub-petioles form the perceptive area for light. The excitation is conducted along the phloem strand of the sub-petiole, and thence through the connected phloem in the petiole. In leaves with four sub-petioles there are, as stated before, four main bundles which reach the motile organ, the pulvinus. There the fibro-vascular bundles apparently fuse, but very fine section of the pulvinus shows lines of separation. In any case, I shall be able to show that nervous strands are physiologically distinct. These terminate in the four effectors, of which two are lateral, the right and the left effectors; the other two are upper and lower effectors. In younger specimens of Mimosa there are two sub-petioles instead of four, and the two nerve strands are continued to the right and left flanks of the pulvinus, the particular innervation being to the right and left effectors respectively. In Helianthus, the right and left nerve pass along the right and left flank of the petiole, which, as we have seen, serves as an extended motor organ. The following results will show that these strands function as distinct nerves :—

Experiment 4.—One electrode was pricked in so as to make contact with the phloem of the right bundle embedded in the petiole; the second contact was made with a distant indifferent point. Electric stimulation of the right vein of the lamina of Helianthus gave rise to electric response of galvanometric negativity, the response being mono-phasic. Application of thermal and chemical stimulus produced similar results (fig. 9).

Experiment 5.—The second electrode was in this case thrust into the nerve of the plant about 1 cm. behind the first electrode. The response is now diphasic, since excitation reached the two points in succession (fig. 9). Discontinuity of the nerve stops the transmitted impulse, as will be seen below.

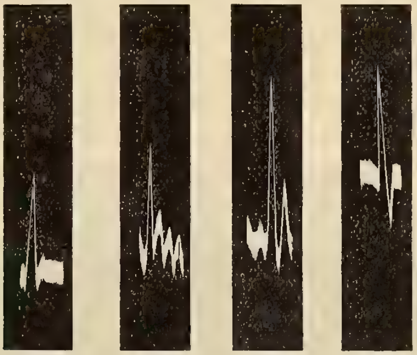

Fig. 9.—Galvanometric record of transmitted excitation in the nerve of Helianthus. The first is in response to electric stimulus, the second and the third to thermal and chemical stimulus. Note in these multiple responses due to strong stimulation. The fourth exhibits diphasic response (see text).

9. *The Directive Action of Propagated Impulse in Heliotropic Leaf-adjustment.*

In Mimosa and in Helianthus I have traced the nervous channels from the receptor to the effector, and showed how the nervous impulse is propagated along definite channels. The most difficult problem that confronts us now is to explain the responsive movement and torsion of the motor organ, by which the expanded leaf surface faces the light. I shall now describe the motor reaction when different parts of the leaf surface are locally stimulated, not only by light, but by diverse modes of stimulation.

(a) *Mimosa pudica.*

Experiment 6.—For this experiment I first took specimens of Mimosa leaf having two sub-petioles. The right sub-petiole was stimulated by feeble tetanising electric shock. The response was by right-handed torsion. The latent period was 2 seconds, and the torsional movement continued for 20 seconds, even on cessation of the stimulus, after which there was a slow recovery, not shown in the record (fig. 10, *a*). The propagated impulse has thus followed its definite path, and reached the right flank of the pulvinus or the right-effector. We saw that the characteristic response of this particular effector is by a right-handed torsion. Thus the same response takes place, whether the effector is directly stimulated or by transmitted excitation. This finds strongest confirmation from the following experiment, where the responsive movement is made to undergo reversal.

Experiment 7.—The left sub-petiole was now stimulated by feeble tetanising shock, as in the last experiment. The response was now by a left-handed torsion (fig. 10, *a*); the nervous impulse now reached the left flank of the pulvinus, or the left-effector, the characteristic response of which is by a left-handed torsion. The leaf may thus be twisted to the right or to the left by alternate stimulation of the two sub-petioles. A feeble tetanising shock should be used for these experiments, since a strong excitation becomes diffused as it reaches the fibro-vascular ring in the pulvinus, and the predominant excitation of the entire lower half would then mask the characteristic effects of the right or the left effectors. As regards leaves with four sub-petioles, we shall presently find that they transmit definite impulses to the four quadrants of the pulvinus, to the right and to the left, to the upper and the lower effectors, thus giving rise to definite reflexes.

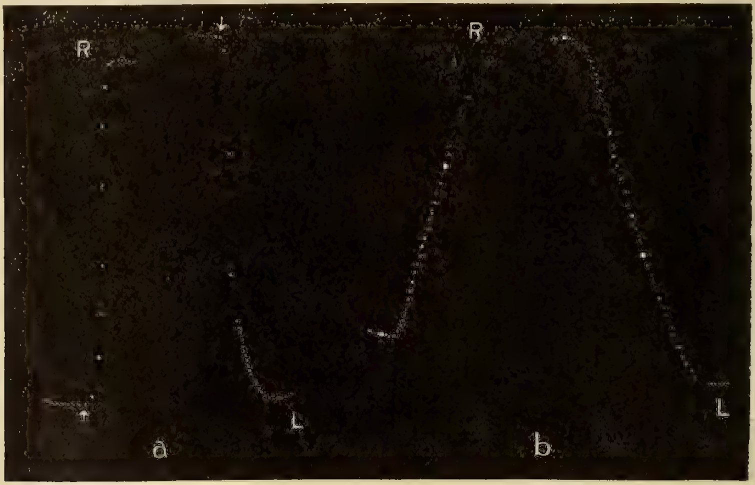

Fig. 10.—Responsive torsion by transmitted excitation in Mimosa under (*a*), electric stimulus; (*b*), under stimulus of light. Note right-handed and left-handed torsions by stimulation of the right and left sub-petioles. Successive dots in (*a*) are at intervals of 2 seconds, and in (*b*) 20 seconds. Note the quick reaction under electric, and slower reaction under photic stimulation.

Experiment 8.—Stimulus of Light.—I next tried the action of stimulus of light on the leaflets of the right sub-petiole; here also the transmitted excitation induced a right-handed torsion. The latent period was, for reasons explained before, longer than in the case of electric stimulation. It should be remembered that the light was applied vertically, and the responsive torsion was such that the amount of light absorbed by the leaflets became reduced by the torsion. Hence it is obvious that it is not the advantage of the plant, but the inevitable physiological reaction, that determines the movement. Stimulation of the leaflets of the left sub-petiole induced a left-handed torsion. When the leaflets of both the sub-petioles were illuminated by vertical light, the two resulting torsions balanced each other. While in this state of dynamic balance, if the intensity of light on one of the sides, say the left, be diminished by interposition of a piece of paper, the balance is at once upset, and we find a right-handed torsion. It is thus seen that equilibrium is only possible when the entire leaf-surface (consisting of the two rows of the leaflets) is equally illuminated; and that would be the case when the surface is perpendicular to the incident light. The dia-heliotropic attitudes of leaves is thus brought about by distinct nervous impulses, initiated at the perceptive region actuating the different effectors.

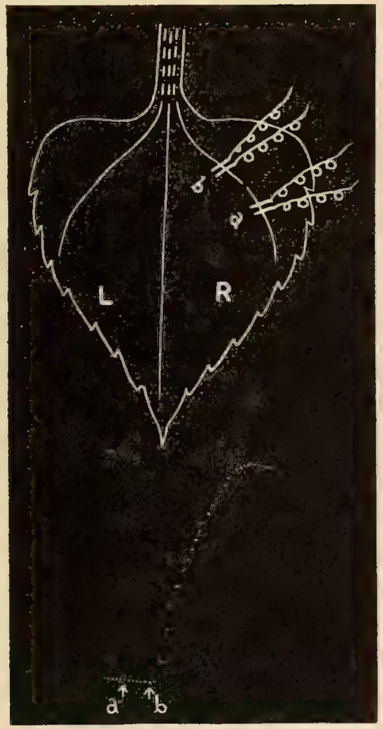

FIG. 11.—The upper figure is a diagram of stimulation of nerve-ending of Helianthus. The record below shows that stimulation beyond the cut gives (*a*), no response; while stimulation at *b*, induces right-handed torsion.

In the case of leaves with four sub-petioles, illumination of the extreme right induces, as already stated, a right-handed torsion; that of the second sub-petiole from the right brings about a movement of erection; the stimulation of the third causes a down movement, while that of the extreme left causes a left-handed torsion. The leaf is thus adjusted in space by co-ordinated action of four distinct reflexes.

(b) *Helianthus annuus.*

Results in every way similar are obtained with leaf of Helianthus. Here we can distinguish three main veins or nerves, which collect excitation from different regions of the lamina.

Experiment 9.—I first tried electric stimulation. The insertions of the electrodes were made in the manner shown in the diagram (fig. 11).

Experiment 10.—Effect of Discontinuity.—A cut is made between *a* and *b*, thus interrupting the continuity of the nerve. Electric stimulation at *a* induced no responsive movement; stimulation at *b* induced, however, the normal response by right-handed torsion (lower record fig. 11).

Experiment 11.—Alternate Electric Stimulation.—The right and left nerve endings in the lamina were stimulated alternately. This gave rise to right-handed and left-handed torsions respectively. In fig. 12, *a*, is given the record of right-handed torsion.

The following experiments will show that photic stimulus induces a reaction which is similar to that of electric stimulus:—

Experiment 12.—Stimulus of Light.—Sunlight was thrown first on the right half and then on the left half of the lamina. The transmitted excitations induced corresponding torsional responses (fig. 12, *b*). A balance was produced when the two halves of the lamina were simultaneously exposed to equal illumination. Here also, as in Mimosa, the heliotropic adjustment is brought about by balanced reactions of the different effectors.

The movement of a dia-heliotropic lamina has been figuratively compared with the movement of the human eye by which it points itself to a luminous object. It is strange that there is more truth in this comparison than was suspected. In describing the rolling of the eyeball Bayliss says (1): "When there are two sets of muscles acting on a movable organ, such as the eye or a part of a limb, in such a way that they antagonise one another, it is clear that for effective performance of a particular reflex movement, any contraction of the muscles opposing this movement must be inhibited. Further, the inhibition of one group must proceed *pari passu* with the excitation of the other group to ensure a well-controlled and steady motion."

Now, in the torsional adjustment of the leaf due to unequal stimulation of the two receptors—the right and left halves of the lamina—let us take the extreme case where one half, say the right, is alone stimulated, either by light or by electric shock. The two effectors for torsional movement, the right and the left, are the responding tissues in the right and left flanks of the petiole. These are actuated by the nervous impulses transmitted along the two conducting strands. When the right half of the lamina is stimulated the transmission of excitation along the conducting strand on the right is detected (Experiment 5) by an electric change of galvanometric negativity, and the corresponding mechanical response of the right effector is, as shown before, by a right-handed torsion. We may next inquire the nature of the transmitted impulse along the left flank of the petiole concomitant with the excitation of the right half of the lamina. It is obvious that a similar excitatory impulse on the left flank (the electric indication of which is galvanometric negativity) would oppose and thus neutralise the particular directive movement. Hence for ensuring a steady directive motion, in response to stimulation of the right half of the lamina, all excitatory impulse to the left flank of the petiole should be inhibited. Further, the directive movement induced by the stimulation of the right half of the lamina would be actively helped if the motor reaction of the left flank of the petiole be of an opposite character to that in the right flank. We found that the right-handed torsion is induced by a differential contraction of the right flank and for concordant effect the reaction of the left flank should be opposite, *i.e.*, a differential expansion. The nervous impulse which actuates the right

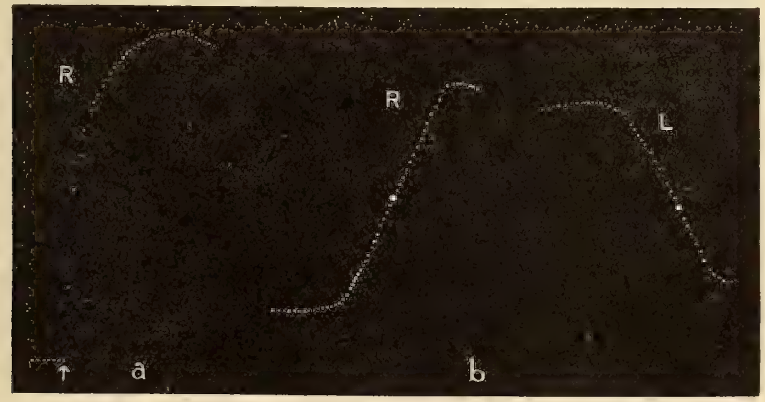

FIG. 12.—Torsional response due to transmitted excitation in Helianthus: (*a*), right-handed torsion due to electric stimulation of the nerve-ending in the right half of the lamina; (*b*), right-handed and left-handed torsions due to transmitted excitations caused by alternate illumination of the right and left half of the lamina. Light was stopped after the thick dot.

effector when the right half of the lamina is alone stimulated, is indicated by galvanometric negativity; for concordant movement under the above condition, the impulse which actuates the left effector should be of opposite sign, *i.e.*, of galvanometric positivity.

I carried out two sets of experiments on the above lines with an identical leaf of Helianthus. First, I carried out the usual experiment of the electric detection of transmitted excitatory impulse. In this, one of the contacts was made with the right nerve in the petiole, the second being with a distant indifferent point. The nerve endings on the right half of the lamina were electrically stimulated and the transmitted impulse along the nerve gave the usual excitatory reaction of galvanometric negativity. A second pair of contacts were made for detection of transmitted impulse in the nerve of the left flank of the petiole. Stimulation of the nerve termination of the right half of the lamina gave in the left nerve a reaction of galvanometric positivity. In practice stimulus was always applied to the right half of the lamina, and galvanometric connections were made alternately with the right and left nerve. The results were always the same and showed that excitation of a nerve gave rise to an opposite reaction in the contiguous nerve. There is no doubt that these two nervous impulses of opposite signs reaching the antagonistic tissues of the two flanks of the motor organ must be of importance in the co-ordination of the resulting movements.

General Summary.

In certain leaves the heliotropic adjustment is brought about by transmission of nervous impulse to the motor organ. A continuity is shown to exist in the response of "sensitive" and ordinary plants. *Mimosa pudica* is taken as a type of the former, and *Helianthus annuus* of the latter. Mechanical response is brought about in both by the differential excitability of the upper and lower halves of the motile organ. The lower half in both is the more excitable. Local stimulation of the abaxial half of the organ induces an erectile movement, that of adaxial half a more rapid downward movement.

Heliotropic curvature of a stem is due to the joint effects of contractile reaction of the proximal and expansion of the distal side.

The daily periodic movements of the leaves of Mimosa and of Helianthus are essentially similar. The diurnal movement is brought about by the variation of the geotropic action with changing temperature, and by the varying intensity of light. The leaves erect themselves during the fall of temperature from thermal noon at 2 P.M. to about 5.30 P.M. Owing to the rapid diminution of light in the evening the leaves undergo an abrupt fall which continues till 9 P.M. After this the leaves erect themselves, till the maximum erection is attained at 6 A.M., which is the thermal dawn. The movement of the leaves is then reversed and there is a continuous fall till the thermal noon at 2 P.M.

A very important motile reaction in the adjustment of leaves is the torsional response to lateral stimulus. The following is the law which determines the directive movement: An anisotropic organ when laterally stimulated by any stimulus undergoes torsion by which the less excitable side is made to face the stimulus. In a dorsi-ventral organ the upper side is, generally speaking, the less excitable side, and the response of such an organ to lateral stimulus may be expressed in the following simple terms. Lateral stimulation of a dorsiventral organ induces a torsion which is right-handed, when the right flank is stimulated. Left-handed torsion is induced by the stimulation of the left flank.

The effects described above take place by direct stimulation of light. They also take place under transmitted excitation.

The motor organ may be regarded as consisting of four effectors; the response of the right effector is by a right-handed torsion, and of the left effector by a left-handed torsion. The upper and lower effectors respond by rectilinear up-and-down movements.

The nervous tissue in plants was localised by means of the Electric Probe which was made to pass by successive steps through the petiole. The maximum transmitted excitation was localised at the phloem portion of the fibro-vascular bundle. Hence the phloem functions as the nerve of the plant.

Excitation at the receptive region is propagated along a definite conducting channel, which is traced from the receptive area in the lamina to the corresponding effector in the motor region.

In a petiole of Mimosa, provided with two sub-petioles carrying rows of leaflets, stimulation of the right row of leaflets by light gives rise to an excitatory impulse which reaches the right effector and induces a right-handed torsion. Stimulation of the left row of leaflets induces the opposite, or left-handed torsion. When both the sub-petioles are illuminated equilibrium is only possible when the entire leaf surface (consisting of the two rows of leaflets) is perpendicular to the incident light. The dia-heliotropic attitude of leaves is thus brought about by distinct nervous impulses initiated at the perceptive region actuating the different effectors.

In Mimosa with four sub-petioles, illumination of the second sub-petiole induces an up-movement; that of the third sub-petiole a down-movement. The leaf is thus adjusted in space by the co-ordinated action of four reflexes.

Results similar to the above were also obtained with Helianthus.

For the movement of the eye the contraction of the muscle opposing the movement has to be inhibited. In the torsional movement of the leaf it is found that the stimulation of one nerve causes in a contiguous nerve an opposite reaction. The nervous impulses of opposite signs reaching different flanks of the motile organ is thus of importance in the co-ordination of the resulting movement.

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The Ultra-Violet Absorption Spectra and the Optical Rotation of the Proteins of Blood Sera.

By S. Judd Lewis, D.Sc. (Tübingen), B.Sc. (London), F.I.C.

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The earlier part of this investigation was described in a paper entitled "The Ultra-Violet Absorption Spectra of Blood Sera," communicated by Sir William Ramsay, K.C.B., to the Royal Society in 1916 and published in the 'Proceedings' (series B, Vol. 89, pp. 327 to 335).

At the close of the paper, attention was directed to the inadequacy of the sector spectrophotometers then available, and reference was made to one of new design then under construction. In the meantime, a full description of this instrument has been published in a paper entitled "A New Sector Spectrophotometer" by the present writer, in the 'Transactions of the Chemical Society' (1919, vol. 115, pp. 312 to 319), together with figure and diagrams. With this instrument completely satisfactory results have been obtained, and with it most of the work now to be described has been done.

The earlier work had reference to serum as a whole; and as foreshadowed in the paper cited, the later effort has been directed to a study of the proteid components of serum and their individual influence on the spectrum. For this, the Beit Research Fund Committee of the British Homœopathic Association have again given generously the necessary financial aid out of the funds placed at their disposal by Mr. Otto Beit for purposes of scientific research.

A search in the literature has failed to reveal any information on the subject, so that in all parts of the work it was necessary to break new ground. Two papers on "Ultraspectroscopic Studies in Blood Serum," one by T. Tadokoro and the other by T. Tadokoro and Y. Nakayama, appeared in America in the 'Journal of Infectious Diseases' for January, 1920 (vol. 26, pp. 1 to 7, and 8 to 15), and recall the subject matter of the first paper cited, but they do not in any way anticipate the present communication.

It has already been observed that the absorption band of serum is caused entirely or almost entirely by the proteins contained, and it became a matter for inquiry as to whether the albumin, pseudo-globulin and eu-globulin were similarly or variously absorbent of ultra-violet light.

The necessary preliminary to a spectroscopic examination of these components was to separate them in a pure state, and to devise means for determining the concentration of the solution employed. This proved an unexpectedly difficult task, partly because of the confused state of the information available and partly because of the necessity of employing solutions perfectly free from preservatives and other substances capable of affecting the spectrum-absorbing power of the solutions. In the end there was no alternative to relying on one's own discretion and to devising the details of the processes of separation and purification. Great care was exercised with a view to purity and constancy of product.

In formulating the processes, much attention was paid to those published by Hardy, Hartley and Haslam, but more particularly to the researches of Dr. Harriette Chick on the physical conditions which control the precipitation of the proteins of serum, published in 1913 and 1914 in the 'Biochemical Journal.'

Method of Separating the Proteins.

In the processes of separation ammonium sulphate was practically the only reagent employed. The purest qualities obtainable commercially were carefully tested for organic matter, as traces of this would affect the absorption spectrum, and the best specimens were selected.

The manner of procedure was in principle such as that usually followed, depending on suitable application of various concentrations of ammonium sulphate; but numerous details were carefully studied, and amongst these the following may be mentioned. It was found desirable to employ fairly large quantities of material, such as two litres, of the clear serum and at each stage to work at first with solutions of ammonium sulphate, which were as precisely as possible of full, one-half or one-third saturation, and then, when required, to add small but known excesses. The albumin precipitates were dissolved in such a quantity of water as to produce a 14 or 16 per cent. solution, and reprecipitated, the first time by the addition of specially recrystallised ammonium sulphate, and on subsequent occasions by the aid of ammonium sulphate and a very small quantity of acetic acid, and left to stand over for some days to allow the precipitate to become micro-crystalline. With horse serum the best crystals were obtained after the fourth precipitation. With human serum, the particles did not form cystals, but they exhibited a well-defined uniformity of shape, suggestive of an approach to a radiate crystalline structure.

The mixed globulins were precipitated three times, and then separated from one another by dissolving in sufficient water to produce an approximately 2 per cent. solution, and precipitating by increasing the ammonium sulphate concentration to one-third saturation.

The pseudo-globulin was freed from accompanying eu-globulin by slightly increasing the ammonium sulphate to nearly 36 per cent. saturation and filtering, and then by adding four small equal quantities of saturated ammonium sulphate solution, and allowing to stand after each addition, so that the final concentration was nearly but not quite 37 per cent. saturated, with a view to removing any remaining eu-globulin. After filtration, the pseudo-globulin was reprecipitated by increasing the ammonium sulphate to one-half saturation, and collected for use.

The eu-globulin was reprecipitated by ammonium sulphate four times from dilute solutions. With ascitic fluid, a small amount of a brown jelly-like substance sometimes accompanied the eu-globulin. When this was the case, the impure eu-globulin was dissolved as completely as possible in a small quantity of water and then saturated ammonium sulphate solution was added, until a small but considerable precipitate was formed, stirred well and filtered. The jelly-like substance remained on the filter together with a small quantity (probably 20 or 30 per cent.) of the eu-globulin. The filtrate was then quite clear, and was treated as an ordinary solution of eu-globulin. Subsequent precipitations usually gave no trouble.

Optical Rotation of the Proteins.

It was at first assumed that each protein would have a constant rotatory power, and that observations of the specific rotations would settle the question of purity. However, the literature of the subject revealed the widest disparity of figures for the same protein, and as reliable data did not appear to be available, recourse was had to determining the concentrations of the solution by chemical means and to taking advantage of the opportunity which the principal work afforded of determining the specific rotations of the several proteins. In view of the separations having been carried out with such thoroughness, the figures should be fairly accurate and reliable.

There is probably a normally definite specific rotation for each of the globulins, although experimental results do not favour this view with regard to the albumins. On the other hand, one must take into consideration such cases as that of the pseudo-globulin from specimen No. 201. The specific rotation was determined twice on two entirely independent solutions of different concentration, the concentrations having to be ascertained separately by chemical means. The two results are −43·26° and −43·82°, whereas the adopted figure for other pseudo-globulins is —46°. This seems to show that if there is a normal value, there are specimens having abnormal values. A study of the figures as a whole leads to the conjecture that a given specimen may be pure in the chemical sense, but consist of a mixture of optical isomers of the protein. A closer examination of the data reveals many irregularities not apparent at first sight. For example, the two most recent and best specimens of pseudo-globulin from the horse had the specific rotations −52·06° and −52·17°, while an earlier specimen gave −49·50°. Corresponding specimens of human origin gave −43·5°, −46·97°, −47·66°, and −45·35°, the first three having been separated from ascitic fluid and the last from normal serum. There is thus exhibited a well-marked differentiation between the rotation of the human, which may be taken as −46°, and that of the horse, which may be taken as −52°.

Similarly with eu-globulin: the figures for the two best and most recent specimens of horse are −43·03° and −43·04°, and for an earlier one −40·98°. The human gave −50·24°, −49·12°, −47·13°, and −47·89°, the first three referring to the protein separated from ascitic fluid and the last from normal serum.

From these one may adopt −43° for eu-globulin from horse and −48° for the human.

With albumin the results fluctuated considerably. For horse −57·40° is the only figure available. For human, −65·36°, −64·43°, −55·05°, −59·14°, −50·58°, −54·83°, the first four referring to albumin separated from ascitic fluid, and the last two to that from normal serum.

The rotations were observed with solutions containing a little ammonium sulphate. By experiment it was ascertained that the change of rotation on varying the concentration of the ammonium sulphate is so small as not appreciably to affect the specific rotations found. The effect under the prevailing conditions of experiment is not likely to be greater than ±0·2 on the specific rotation.

Spectrophotometry of the Solutions.

Strong solutions of the proteins were obtained by dissolving the purified precipitates described above in water, and their concentrations were ascertained by determining the total solids and the ammonium sulphate in the solution, and taking the difference as protein. This method gave constant and apparently satisfactory results. In every case the work was done in duplicate, and sometimes in triplicate.

The strong solution was polarised with the object of determining the specific rotation, and suitably diluted with distilled water for use in the spectrophotometer. The concentrations found to work best, that is, such as to exhibit a well-developed band, have been found to be 0·08 per cent. for the albumin, 0·04 per cent. for the pseudo-globulin and for the eu-globulin.

The strengths of the solutions were estimated approximately by means of the polarimeter for immediate use, and corrected later when the chemical figures became available.

The dilution was filled into a 2-cm. observation tube fitted with quartz ends, and a second tube was filled with a solution of ammonium sulphate of approximately the same strength as was the protein solution with reference to this salt: usually this was obtained by diluting saturated ammonium sulphate solution 150 times. The latter tube was used as a blank in the one path of the spectrophotometer, so that the observations made with the tube of protein solution placed in the other path express the spectrum-absorbing effect of the protein only.

The process of spectrophotometry was conducted in the manner indicated in the paper first cited, with all the refinements described in the paper dealing with the new instrument. The series of photographs for each experiment extended over three plates, making a series of fifty-four in all.

The absorption curve is plotted with extinction coefficients as ordinates and wave-lengths as abscissæ. The extinction coefficient is calculated on a 1-cm. layer of a 0·1 per cent. solution of the protein, which, according to Beer's law, is the same as that on a 0·1-mm. layer of a 10 per cent. solution. This corresponds with the protein concentration of serum as nearly as decimal figures permit; serum contains about 8 per cent of proteins. Hence the curves approximate in their values to those already described with reference to serum itself. In order to correlate the two sets of values, either the extinction coefficients of serum must be multiplied by 1·25 or those of the proteins by 0·8.

Each of the protein curves is in general similar in form and character to that of serum (*loc. cit.*), which demonstrates that the band produced by serum is an expression of its protein-content, especially since serum deprived of its protein gives no such band (*loc. cit.*).

It now remains to consider the specimens employed and the curve for each protein in detail. In all, eleven specimens of serum were studied, six derived from the horse and five human. The primary object in employing horse serum was to ascertain the best conditions for separating and purifying the proteins, so that the human serum might afterwards be studied with greater confidence and certainty. The two or three earlier numbers amongst the horse specimens may therefore be regarded as practice numbers, and to that extent the figures for these must be held as less reliable.

The horse serum was as nearly as possible strictly normal, as the first three specimens were supplied as such from a physiological laboratory; and the three later specimens were derived from animals slaughtered for use as human food. The serum was mixed with an equal volume of saturated ammonium sulphate solution within 24 hours of the slaughter of the horse.

The last of the specimens of human serum (No. 205) was declared to be strictly "normal," and was from a case of cerebral hæmorrhage. The other four were selected specimens of ascitic fluid. Each one was quite clear and had the appearance of good serum: No. 200 being the least satisfactory, although that was good.

The three later specimens of horse serum and those of ascitic fluid were all sufficiently large. That of normal human serum was smaller, namely, 250 c.c.; but in this case, by careful manipulation, satisfactory separation and purification of the three proteins were effected.

The experimental data are collated in Tables I to VI, in which the specific rotations are repeated for the sake of easy reference. The results are graphically displayed in the accompanying curves, which have been arranged in two groups, namely, the three proteins from horse serum in the one, and those of human origin in the other. This is convenient, because the curves for the two pseudo-globulins are so very nearly alike that they may be regarded as the one a replica of the other.

The values brought together in the Tables for study and comparison are (*a*) the extinction coefficient at the head of the absorption band at a wave-length of about 2800; (*b*) the extinction coefficient at the point in the band where the light-absorbing power is least at a wave-length of about 2500, that is, in the depression of the curve; (*c*) the difference between (*a*) and (*b*), or the "amplitude" of the band, which shows considerable regularity, and appears to be significant; (*d*) the wave-length of the region of greatest absorption in the band, that is, at the head; (*e*) the wave-length of the point of least absorption in the band, that is at the foot of the curve in the depression.

Table I.—Pseudo-Globulin from the Horse.

Specimen number.	Extinction coefficient.			Wave-length.		Specific rotation.
	Of head at 2800.	Of foot at 2500.	Difference or amplitude.	Head.	Foot.	°
193	1·12	0·43	0·69	2790	2510	
194	1·31	0·52	0·79	2800	2510	
197	1·18	0·46	0·72	2780	2518	
198	2·01	1·41	0·60	2780	2530	−49·50
199	1·29	0·57	0·72	2800	2540	−52·06
203	1·18	0·46	0·72	2790	2500	−52·17
Adopted	1·19	0·47	0·72	2790	2520	−52

Table II.—Pseudo-Globulin (Human).

Specimen number.	Extinction coefficient.			Wave-length.		Specific rotation.
	Of head at 2800.	Of foot at 2500.	Difference or amplitude.	Head.	Foot.	°
200 ascitic fluid	1·26	0·61	0·65	2750	2520	
201 „ „	1·35	0·63	0·72	2780	2528	(*a*) −43·26 (*b*) −43·82
202 „ „	1·46	0·72	0·74	2790	2520	−46·97
204 „ „	1·40	0·67	0·73	2805	2525	−47·66
205 normal serum	1·28	0·58	0·70	2790	2520	−45·35
Adopted	1·35	0·63	0·72	2790	2521	−46

Observations on the Figures for Pseudo-globulin, Tables I and II.

Omitting the earlier numbers, 193 and 194, which represent the first efforts, and 200, which was not a very good specimen, the figures exhibit a remarkable regularity in the amplitude of the absorption curve as expressed by the difference in the extinction coefficients. With one exception (No. 198), they all lie between 0·70 and 0·74. Very little difference between the horse and human specimens is evident. The chief distinction is that the corresponding coefficients are a little higher for the human than for the horse.

One of the most striking features of the work is the discovery that pseudo-globulin has probably identically, certainly almost identically, the same form of absorption band of the same magnitude, whether it is of horse or human origin. This is good evidence of pseudo-globulin being a chemical entity.

Table III.—Eu-Globulin from the Horse.

Specimen number.	Extinction coefficient.			Wave-length.		Specific rotation.
	Of head at 2800.	Of foot at 2500.	Difference or amplitude.	Head.	Foot.	°
193	1·68	0·48	1·20	2800	2530	
194	—	0·53	—	—	2515	
197	—	—	—	—	—	
198	1·44	0·62	0·82	2760	2530	−40·98
199	1·09	0·62	0·47	2770	2530	−43·03
203	1·42	0·89	0·53	2780	2535	−43·04
Adopted	1·42	0·89	0·53	2775	2532	−43

Table IV.—Eu-Globulin—Human.

Specimen number.	Extinction coefficient.			Wave-length.		Specific rotation.
	Of head at 2800.	Of foot at 2500.	Difference or amplitude.	Head.	Foot.	°
200 ascitic fluid	1·37	0·71	0·66	2750	2630	−50·24
201 „ „	1·46	0·78	0·68	2770	2510	−49·12
202 „ „	1·39	0·67	0·72	2800	2540	−47·13
204 „ „	1·53	0·85	0·68	2805	2525	
205 normal serum	1·52	0·89	0·63	2790	2530	−47·89
Adopted	1·51	0·85	0·66	2795	2530	−48

Observations on the Figures for Eu-globulin, Tables III and IV.

The curves for the two eu-globulins are so similar in their general form and magnitude to those for pseudo-globulin as to leave no doubt of a close chemical relationship between the two groups, but the minor quantitative distinctions are too great and too well-defined to allow of their being regarded as mere varieties of the same substance.

At the present time, it is a matter of great interest to discover any fundamental differences between pseudo-globulin and eu-globulin. Dr. Harriette

Chick considers the two globulins to be the one a modification of the other.* The results of the present inquiry may, on the whole, be held to support this view, but they do certainly indicate also that there is some important difference between them, which is borne out by the two following considerations:—

(*a*) The amplitude figure is much greater for the pseudo-globulin than it is for the eu-globulin.

For pseudo-globulin this figure is 0·72 for both horse and human; for eu-globulin it is 0·53 for horse and 0·66 for human. Therefore, with both varieties, there is a marked difference between the two globulins.

Also, for both horse and human proteins, the curve for pseudo-globulin is very slightly broader than that for eu-globulin; this is so for the human more than for the horse.

(*b*) The intensity of the selective absorption is much greater for the eu-globulin than it is for the pseudo-globulin, as shown by the magnitudes of the extinction coefficients.

For horse, the figures for eu-globulin are 1·42 for the head and 0·89 for the foot, mean 1·16, against those for pseudo-globulin, which are 1·19 for the head and 0·47 for the foot, mean 0·83, giving an excess in favour of the eu-globulin of 0·33, or 39 per cent.

For human, the corresponding figures for eu-globulin are 1·51 for the head and 0·85 for the foot, mean 1·18, against those for pseudo-globulin, which are 1·35 for the head and 0·63 for the foot, mean 0·99, giving an excess in favour of the eu-globulin of 0·19, or 19 per cent.

In passing, it may be observed that both horse and human pseudo-globulin exhibit the same amplitude in the band, namely, 0·72, and that the bands for both horse and human eu-globulin have nearly the same mean values for the extinction coefficient, namely, 1·16 and 1·18.

Although the problem is not yet solved, it may be said that the spectroscopic phenomena are in harmony with the view expressed by Dr. Chick, that eu-globulin is a protein-lipoid complex resulting from the interaction of pseudo-globulin with a minute proportion of a lecithin.

Observations on the Figures for Albumin, Tables V and VI.

The extinction coefficients of albumin contrast strongly with those for either of the two globulins. The amplitude value is only 0·36 for the horse or 0·23 for the human, instead of about 0·7, which was found for the two globulins.

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* 'Biochem. Journ.,' vol. 8, pp. 404–420 (1914).

Table V.—Albumin from the Horse.

Specimen number.	Extinction coefficient.			Wave-length.		Specific rotation.
	Of head at 2800.	Of foot at 2500.	Difference or amplitude.	Head.	Foot.	°
193	—	—	—	—	—	
194	0·55	0·32	0·23	2780	2520	
197	1·60	1·21	0·39	2780	2540	
198	1·42	0·97	0·45	2770	2540	−57·40
199	0·89	0·68	0·21	2770	2545	
203	1·19	0·84	0·35	2800	2550	
Adopted	1·20	0·84	0·36	2785	2545	

Table VI.—Albumin—Human.

Specimen number.	Extinction coefficient.			Wave-length.		Specific rotation.
	Of head at 2800.	Of foot at 2500.	Difference or amplitude.	Head.	Foot.	°
200 ascitic fluid	1·05	0·86	0·19	2770	2575	−65·36
201 ,, ,,	0·67	0·37	0·30	2765	2540	−64·43
202 ,, ,,	0·70	0·38	0·32	2795	2540	−55·05
204 ,, ,,	0·58	0·33	0·25	2780	2540	−59·14
205 normal serum	*0·70	0·51	0·19	2780	2555	−50·58
	†0·66	0·45	0·21	2790	2538	−54·83
Adopted	0·68	0·45	0·23	2783	2540	

* 1st crop. † 2nd crop.

The distinction is not in the amplitude alone. The extinction coefficients at the head and foot of the curve in the horse series are fairly high, while in the human series they are exceptionally low, so that the horse albumin is well differentiated from the human albumin.

On tabulating the adopted figures for the amplitude of the band, the wave-length of the head and the wave-length of the foot, and extracting their means as shown in Table VII, one is impressed with the uniformity in the wave-length of the head, which is very nearly the same for the three proteins, and yet clearly not identical for pseudo-globulin (2790) and albumin (2784), while the two varieties of eu-globulin provide extremes at 2775 and 2795. The wave-lengths of the foot for the three proteins are also nearly the same, although again clearly differentiated: 2521 for pseudo-globulin, 2531 for eu-globulin, and 2543 for albumin. The differences are small, but there is no reason for doubting that they are real. The approximate

Table VII.—Comparative Observations on the Results.

	Amplitude.	λ for head.	λ for foot.
Pseudo-globulin—			
Horse	0·72	2790	2520
Human	0·72	2790	2521
Mean	0·72	2790	2521
Eu-globulin—			
Horse	0·53	2775	2532
Human	0·66	2795	2530
Mean	0·60	2785	2531
Albumin—			
Horse	0·36	2785	2545
Human	0·23	2783	2540
Mean	0·30	2784	2543

identity of the wave-lengths signifies the close similarity of chemical constitution for the three proteins, while the small differences, which are well substantiated so far as the present series of experiments goes, may possibly be significant of differences in the nature of the subsidiary groups present in the respective molecules. On the other hand, the amplitudes, which are respectively 0·72, 0·60 and 0·30, reveal differences which are too great to be lightly set aside.

It remains, then, that it is mainly in the magnitude of the extinction coefficients that the differences among these three proteins find expression. This has already received some attention in the comparison of eu-globulin with pseudo-globulin; but, with albumin, the divergence from the apparently well-defined pseudo-globulin is much greater, and not of quite the same order; and, further, there is no doubt as to horse albumin being quantitatively distinct from human albumin.

If the ratio of the adopted extinction coefficients for the two varieties of albumin be taken, we get

	Head of band.	Foot of band.	Amplitude.
$\frac{\text{Horse}}{\text{Human}}$	$\frac{1·20}{0·68} = 1·77$	$\frac{0·84}{0·45} = 1·87$	$\frac{0·36}{0·23} = 1·57$

or, if the figures for the two specimens of human albumin from "normal" serum alone be taken, we get

$$\frac{1·20}{0·68} = 1·77 \qquad \frac{0·84}{0·48} = 1·75 \qquad \frac{0·36}{0·20} = 1·80$$

This regular ratio for the several parts of the absorption curve would ordinarily signify a corresponding difference in the concentration of the substance in solution, but, inasmuch as the percentage concentration of the albumin was the same for all the solutions, both in the experiment and in the ultimate calculations, some other explanation must be found. The work of Kober* and others has shown that aromatic amino acids exhibit selective absorption, while the absorption spectra of several of the aliphatic amino acids and the simpler polypeptides exhibit no selective absorption, and that even 40 mm. layers of 0·05 per cent. solutions of some of the latter show no general absorption beyond a wave-length of 2500. In view of the way in which the proteids are built up of amino-acids and similar groups, some exerting selective absorption and some not, and of the difficulty in accounting for some of the properties of the proteins when viewed as chemical entities, it is not unreasonable to regard them as products resulting from an essentially physical association of substances comparable with, but not so chemical as, the association of a salt with its water of crystallisation. It may then be assumed that the aggregate composing human albumin may result from such a union of a substance comparable with that composing horse albumin with a substance or substances possessing no selective absorption. Such an hypothesis of physical association gains some support from the fact that five out of six of the amplitude values, 0·72, 0·72, 0·53, 0·66, 0·36, 0·20, are approximately simple multiples of 0·18, and from the view held by Prof. F. Gowland Hopkins† that even the apparently well-defined crystallised egg albumin is composed of several proteids, and that much the same may be said of serum albumin. It is conceivable that each of the proteins now studied is an aggregate resulting from the physical association of a proteid substance exhibiting selective absorption with various but definite proportions of such simpler bodies as those described above as exerting only general absorption. Had the results now recorded been anticipated, it would have been instructive to determine in each specimen the yield of phenylalanine, tryptophane and similar products exhibiting selective absorption, to see whether the proportion was correlated with the extinction coefficient or not. It would have given some indication of how far the association of the groups is physical or chemical.

Moreover, if all the curves be re-drawn so as to have the same amplitude, say 0·72 or 1·0, very little difference will be observed in their form. However, the two albumins would be distinguished from the globulins by the general absorption being slightly greater, extending to about 2450 at the extinction coefficient of the head of the band against 2420 for any of the globulins, and to this one may perhaps attribute the higher figure, 2543, for the wave-length of the foot of the curve in the albumins. The inference from this is that the general absorption of the simpler body in albumin is somewhat greater than it is in the globulins. It is interesting also to observe that the absorption curve of pseudo-globulin which has the greatest amplitude proved to be the most constant of all in the course of the experiments now described, and was the same whether for horse or man, and would thus appear to be a much more definite substance than either eu-globulin or albumin; it may even be a chemical entity.

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* 'J. Biol. Chem.,' vol. 22, pp. 433–441 (1915).

† 'J. Physiol.,' vol. 25, pp. 306–330 (1900).

On the other hand, it is possible that the differences between the absorption spectra of the proteins are essentially chemical in their significance, and then they are not capable of such simple explanation.

The Absorption Curves.

It will be seen that at the foot of each column in the Tables I to VI, an "adopted" value is given. This value is not the arithmetic mean of the experimental figures, but the value deemed to be the best after considering all the circumstances, giving great weight to the most successful experiments and little or nothing to those of doubtful value. Hence, the factors for the later and better specimens differ only very slightly from the adopted values as shown in the Tables.

With these values a mean curve has been drawn in the following way. First, the band for each separate specimen of protein, that is the part of the curve covered by the "difference" or "amplitude" values was divided at the proper extinction coefficients into ten equal parts and the points of division were designated "position 0" at the extinction coefficient of the head, "1st position" one-tenth of the way down, "2nd position" two-tenths of the way down, and so on until the "10th position" is at the extinction coefficient at the foot of the depression. All the curves were then re-drawn to the scale expressed by the adopted amplitude or "difference" of extinction coefficient.

Next, the wave-length at each "position" on the curve was read from the curve for each specimen, and a value adopted. The adopted values are set out in Table VIII and have been used in plotting the curves (see p. 192).

It should be observed that although the mean curves show points plotted at only about twelve extinction coefficients, and at similar positions for all the specimens, absorption spectra were photographed at 50 or more extinction coefficients, and hence each original curve has a corresponding number of points plotted, similar to those plotted in the curve figured in the first paper.

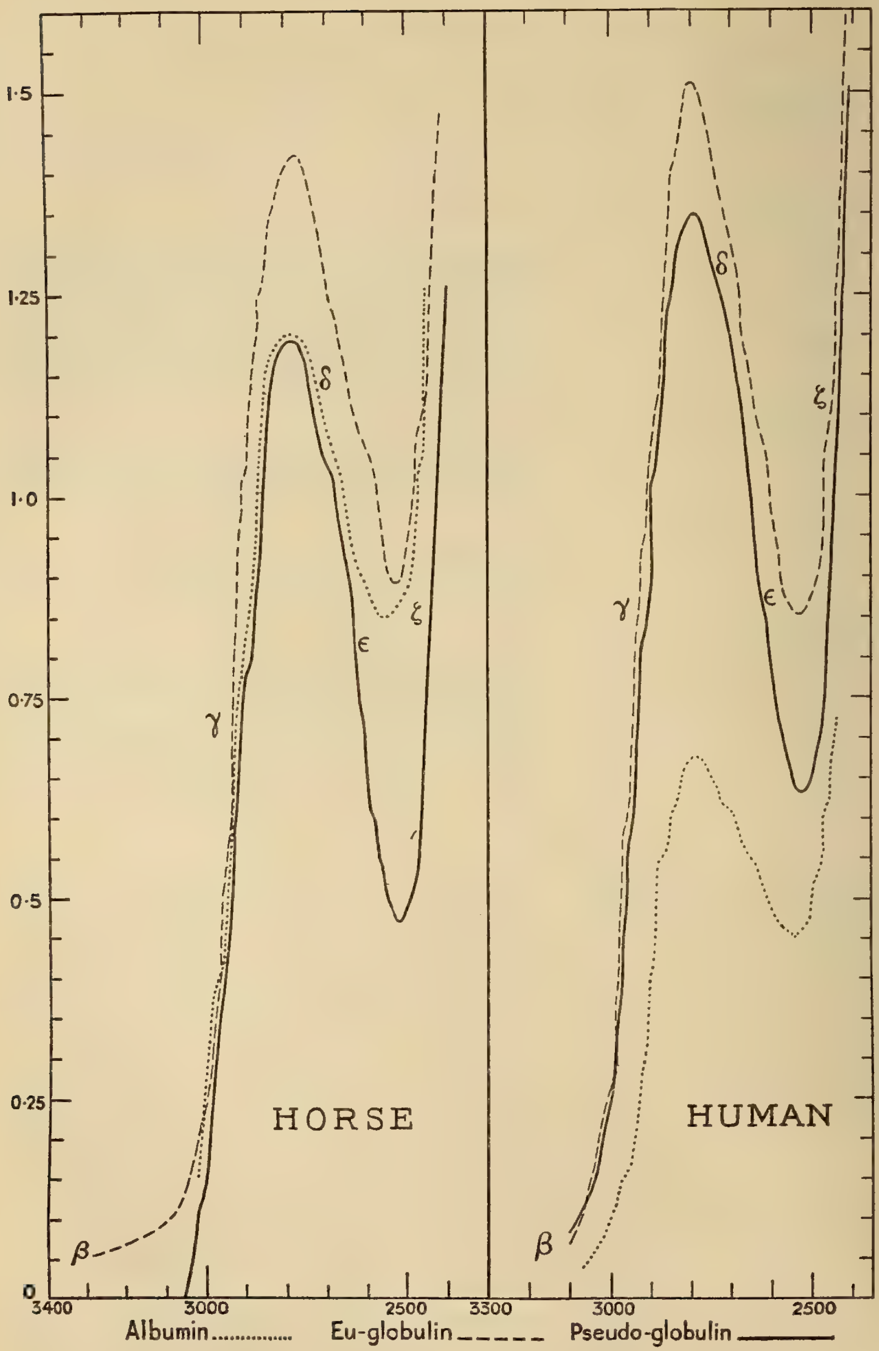
Table VIII.

Protein and variety.	Section.	Position.							
		0.	1.	2.	4.	6.	8.	9.	10.
		Adopted wave-lengths.							
Pseudo-globulin—									
Horse	γ	2790	2847	2860	2875	2900	2930	2935	2948
	ε	2790	2735	2710	2670	2630	2590	2562	2520
	ζ	2410	2415	2420	2430	2445	2460	2475	2520
Human	γ	2790	2845	2858	2864	2895	2929	2935	2943
	ε	2790	2744	2710	2686	2635	2595	2572	2521
	ζ	2418	2423	2428	2430	2445	2461	2480	2521
Eu-globulin—									
Horse	γ	2775	2825	2849	2865	2885	2905	2920	2925
	ε	2775	2740	2718	2675	2635	2579	2559	2532
	ζ	2420	2435	2437	2443	2460	2480	2490	2532
Human	γ	2795	2830	2850	2864	2880	2905	2920	2930
	ε	2795	2760	2730	2680	2648	2605	2578	2530
	ζ	2422	2424	2426	2437	2450	2469	2480	2530
Albumin—									
Horse	γ	2785	2840	2952	2863	2870	2880	2885	2897
	ε	2785	2730	2720	2688	2660	2620	2592	2545
	ζ	2456	2457	2460	2465	2475	2490	2508	2545
Human	γ	2783	2815	2825	2850	2871	2880	2882	2885
	ε	2783	2755	2730	2685	2645	2610	2575	2540
	ζ	2450	2455	2457	2465	2480	2495	2510	2540

The Characteristics of the Mean Curves.

The curves are divided into Sections α, β, γ, δ, ϵ, ζ, as before (*loc. cit.*). Among the more remarkable characteristics are the curious form of the head, Section δ, and the step-like prominences or "steps" to which reference was made in the first paper. It was hoped that the analysis of the serum into its several proteins would have revealed the origin of these steps by showing them to be irregularities due to imperfect superposition of the bands of two or more proteins, but that expectation has not materialised. On the other hand, these characteristics appear in both the globulins with unerring regularity, and with but little variation, in either magnitude or position. In albumin the "steps" are not so numerous, and they are not much in evidence above the tenth position. Below that two large "steps" are evident.

It is difficult to say whether their positions, that is, their extinction coefficients, are precisely the same for all specimens of a given protein or not. They repeat themselves with sufficient regularity to suggest that the positions may be definite approximately as shown; but on the other hand, the variation is too great to attribute it to experimental conditions. The general conclusion



is that the protein varies, since not only do the positions change more or less, but also sometimes the number of steps in a certain section.

Section β has been developed in only a few cases. The most notable observation is that the absorption in this region is usually very small. The curves and especially the spectrum photographs demonstrate in several cases that the maximum extinction coefficient is considerably less than 0·01. It is certainly very much less with the proteins than it is with serum.

Section γ presents no peculiarities, save only that the sweep is broken by four or five "steps," especially by a prominent one not far from extinction coefficient 0·25.

Section δ is found to characterise all the proteins more or less, although not always in so pronounced a manner as many sera. The mean curve does not exhibit this quite so well as the individual curves do, as the construction of the mean curve has a smoothing-out effect. With human eu-globulin, the head of the curve is narrowed from both sides.

Section ϵ is usually the most irregular in form for any given specimen, and it is here that there is most disturbance in the wave-length. This is partly, but not wholly, accounted for by the "steps."

Section ζ defines the limit of the general absorption, and shows very little variation. Only slight "steps" occur occasionally. The most notable feature is that it bends sharply towards the red at the bottom where it joins the Section ϵ.

Summary.

1. The primary object of the investigation was to ascertain the contribution made by each protein constituent of serum to the ultra-violet absorption spectrum curve of blood serum.

2. It has been shown that the absorption curve of pseudo-globulin is constant, and is the same for both the horse and human varieties.

3. The absorption curve for eu-globulin differs considerably from that for pseudo-globulin in extinction coefficients, but not in general form. This favours the view that the differences between pseudo-globulin and eu-globulin do not result from differences in the structure of the chemical molecule.

4. The absoption curves for the horse and human varieties of albumin have been shown to be the same, except for a constant ratio in their magnitudes, and this difference may be due to the physical, or possibly chemical, association of an aggregate, possessing little or no selective absorptive power—for example, an aliphatic amino-acid or a polypeptide—with the principal or absorbing aggregate.

5. The close similarity in form of all the curves when corrected to a common amplitude and the fact that the amplitudes are nearly all simple multiples of a common factor, point to similarity of constitution amongst these proteins and to a variable "concentration" of the active group.

6. Comparisons between the absorptions of the proteins of human serum reveal absorption bands for the horse somewhat greater in dimensions than those for the human.

7. The optical properties of the proteins of serum have been investigated with fairly satisfactory results.

8. Processes for the separation and purification of the proteins have been elaborated.

The Colouring Matter of Red Roses.

By Geoffrey Currey.

(Communicated by Prof. F. Keeble, F.R.S. Received August 12, 1921.)

An examination of the petals of the red rose "George Dickson," has shown that the anthocyan pigment contained therein is the cyanidin glucoside, cyanin. It is present to the extent of about 9–10 per cent. by weight of the dried petals, and exists in the petals as an oxonium salt (*i.e.*, in combination with a plant acid). A yellow glucoside sap-pigment also occurs in the same flowers, but beyond the fact that it has been shown to be capable of producing an anthocyan, by reduction, and that it is not a glucoside of the flavonol myricetin, it has not been further identified, on account of the small quantity present. Further work may show it to be a glucoside of quercetin, and corroborate the work of Dr. Everest,* on the purple-black viola, in which it was shown that an anthocyan ("violanin") and the flavonol glucoside from which it could be produced, by reduction (a glucoside of myricetin), are present, side by side, in the same flowers. This would be additional evidence in favour of the hypothesis that "anthocyans are produced, in nature, by the reduction of the flavonols." It is interesting to note that this rose, grown in Australia, contains the same colouring matter as was isolated by Willstätter and Nolan† from the rose known as "Rosa Gallica," grown in Europe, and shows how widely these colouring matters are distributed in nature.

The rose "George Dickson" was chosen for this investigation on account of its deep red colour, which would indicate a fairly large percentage of the anthocyan pigment. The flowers from which the petals were gathered were grown by Mr. G. Knight, at his nursery, Parramatta Road, Homebush, and his generosity in supplying me with sufficient material enabled the work to be successfully accomplished.

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* 'Roy. Soc. Proc.,' B, vol. 90, p. 255 (1918).

† 'Annalen,' vol. 408, p. 1 (1915).

For the isolation of the anthocyan pigment, the methods used by Willstätter and Nolan, in their investigation of the "Rosa Gallica," were adopted, while the examination of the flavonol pigment was carried out on somewhat similar lines to that used by Dr. Everest in his examination of the viola.

EXPERIMENTAL.

Isolation of the Anthocyan Pigment.

100 grm. of the petals (which had been first air-dried in the shade, at room temperature, and finally over concentrated sulphuric acid), were allowed to stand in a closed vessel, with about 300 c.c. of methyl alcohol, containing 2 per cent. of concentrated hydrochloric acid (to prevent pseudo-base formation), for about twenty-four hours; the mass was then pressed, to obtain as much extract as possible, and the residue treated with a further quantity of methyl alcoholic hydrochloric acid. After standing some hours, this was filtered, with suction, and as the extraction was still incomplete, the residue was again extracted with the same solvent. The residue after filtering and washing possessed but a pale pink colour, and further extraction was deemed unnecessary. The filtrates and washings were united; they possessed a fine, deep red colour, with a bluish-violet tinge at the edge of the solution. The combined filtrates and washings were poured into about three times their volume of ether, well agitated, and allowed to stand for some hours. Practically all the anthocyan pigment separated out as a dark brown, gummy mass, from which the supernatant solution could be readily decanted.

The crude anthocyan pigment was redissolved in methyl alcoholic hydrochloric acid, and reprecipitated with ether (about two and a half times the volume of the alcoholic solution). After standing some hours, to allow precipitation to be as complete as possible, the ether-alcohol solution was decanted off and the precipitate allowed to stand for twenty-four hours in contact with a mixture of methyl alcohol and glacial acetic acid (to remove impurities capable of being hydrolysed or acetylated), and finally collected on a filter, washed with a small quantity of methyl alcohol (containing 1 per cent. hydrochloric acid), and air-dried.

The dark brown powder thus obtained was dissolved in boiling water, an equal volume of ethyl alcohol (containing 3 per cent. hydrochloric acid) added, and the solution allowed to cool; the colouring matter separated out in the form of dark brown leaflets, possessing a golden reflex. These were collected and air-dried. For identification purposes the anthocyanin chloride thus obtained was examined for the following properties, viz., crystalline form, colour, and reflex; colour changes on the addition of ferric chloride to aqueous and alcoholic solutions; colour of solutions in aqueous acid and alcohol; colour changes with alkalies; behaviour with Fehling's solution (hot and cold); colour of precipitate with lead acetate; behaviour with sodium bisulphite; behaviour with zinc and dilute acid; solubility in ethyl alcohol at 19° C.; solubility in aqueous hydrochloric acid (1 per cent. and 1·5 per cent.) at 20° C.; and the distribution of the pigment between amyl alcohol and dilute aqueous acid.

On comparing these properties with those given by Willstätter and Nolan for cyanin chloride, they were found to be identical; the anthocyan pigment of the red rose "George Dickson" is, therefore, the di-glucoside cyanin.

Hydrolysis of the Cyanin Chloride.

The hydrolysis was carried out by boiling a small quantity of the glucoside pigment with 20 per cent. hydrochloric acid for three minutes; on standing, the sugar-free pigment separated in small needles (not long ones, as obtained by Willstätter), possessing a metallic lustre. On examination these were found to possess the same properties as described by Willstätter and Everest for cyanin chloride.

Examination of the Yellow Sap-Pigment.

The ether-alcohol liquor, decanted from the anthocyan precipitate, was shaken with powdered calcium carbonate, and, after being allowed to stand for a short time, filtered. The filtrate, which possessed a clear, deep yellow colour, was concentrated to a small bulk and treated in the following manner:—A portion was evaporated to dryness and the residue extracted with ether; the ether solution was washed with dilute hydrochloric acid, to free it from traces of anthocyan pigment, filtered, and the filtrate shaken with dilute sodium carbonate solution, which became yellow in colour. Having shown that this alkaline solution contained the pigment in the form of a glucoside, the remainder of the concentrate was poured into water and boiled, to expel the small quantity of alcohol still present; the aqueous solution was then boiled with hydrochloric acid, to hydrolyse the glucoside present, allowed to cool, and extracted with ether. The ether extract was washed several times with aqueous acid, of different strengths, to ensure the removal of traces of anthocyanidin, and finally filtered. The filtrate was then shaken with dilute alkali, when the pigment passed into the alkaline layer, which assumed a deep yellow colour (the absence of a green colour here shows the absence of myricetin), which rapidly became dark brown on exposure to air (a possible explanation of this oxidation is given later). The alkaline solution was acidified, extracted with ether, and the ether extract evaporated to dryness. The residue, on being dissolved in absolute alcohol, to which a small quantity of hydrochloric acid had been added, yielded, on the addition of a small piece of magnesium ribbon, a fine red coloration, similar to an alcoholic solution of cyanidin chloride. It is possible that the yellow pigment present in the petals is a quercetin glucoside, but it was not possible to make further tests, as the quantity of pigment available was very small. In order to add additional weight to the assumption that a glucoside of quercetin is present, the above-mentioned tests were carried out with a known glucoside of quercetin, viz., "myrticolorin." A small quantity was boiled with acid and hydrolysed, and the resulting sugar-free pigment "quercetin" treated, in alcoholic solution, with hydrochloric acid and magnesium ribbon in the same manner as with the sugar-free pigment from the rose, exactly similar results were obtained. Tests made with both pigments in a glucoside form also agreed. It is hoped that further work on this rose will result in sufficient of the yellow flavonol sap-pigment being isolated to confirm its identity, by means of derivatives. For the sample of "myrticolorin" used in the above tests, I am indebted to Mr. H. G. Smith, of the Technological Museum, who very kindly supplied me with it; it is a rhammo-glucoside of quercetin, and was isolated from eucalyptus leaves.

Possible Cause of the Rapid Darkening of the Alkaline Solution of the Yellow Sap-pigment from the Rose.

Although it is the property of the flavonols to undergo oxidation on exposure to air in alkaline solution, the very rapid oxygen absorption of the solution previously mentioned would appear to be due to some other cause. It was thought that this might possibly be due to tannin matter. An examination of the petals of the rose revealed the fact that both pyrogallol and catechol tannins were present, the former (probably gallo-tannin), being present in the greater quantity. The alcoholic extract of the petals would contain the tannin matter in solution, and the boiling with acid, to hydrolyse the glucosides, as previously mentioned, would also hydrolyse the gallo-tannin, with the production of gallic acid; the subsequent extraction of the acid solution with ether, to obtain the hydrolysed glucosides, would also extract the gallic acid, and this would accompany them into the alkaline solution; the presence of a small quantity of gallic acid would be quite sufficient to cause a rapid darkening of the solution, on exposure to air.

The Kata-thermometer as a Measure of Ventilation.

By LEONARD HILL, F.R.S., H. M. VERNON and D. HARGOOD-ASH.

(Received August 20, 1921.)

(From the National Institute for Medical Research, Hampstead.)

In two papers previously published, one by Leonard Hill, O. W. Griffiths and M. Flack,* and the other by Leonard Hill and D. Hargood-Ash,† the kata-thermometer was described in detail, and formulæ were given connecting the heat loss with temperature, wind velocity and vapour pressure. Various discrepancies were found to occur, however, and seeing that the kata-thermometer has become recognised as a measure of ventilation the whole matter has now been carefully reinvestigated. Large wind tunnels such as those at the National Physical Laboratory, which were not available during the war, owing to the urgency of aeroplane work, were now at our disposal.

Experimental Work.

To obtain known wind velocities for the experimetal work two methods were adopted, that of the "wind tunnel" where the air is drawn through a long tunnel by means of a propeller at one end, and that of the "whirling arm" where the "kata" is made to move through the air on a revolving arm. The wind tunnel work was carried out in the engineering department at the National Physical Laboratory by kind permission of Dr. T. E. Stanton, where Miss D. Marshall gave us valuable assistance in the determination of wind velocities; also at East London College, where Dr. N. A. V. Piercy was good enough to allow us the use of the tunnel and to help us in determining the wind velocities.

Observations with the whirling arm method were taken at the Physiological Laboratory, Oxford, and at the National Institute for Medical Research, Hampstead. The observations at Oxford were made: (i) in a large room, all the doors and windows of which were closed, and air currents were further reduced by surrounding three sides of the table on which the apparatus stood by screens covered with several thicknesses of brown paper. The fourth side had to be left open, so as to admit of light, and of a point from which to view the "kata." Air draught from below was prevented by means of horizontal screens placed on the table supporting the apparatus. A moving air current of known velocity was obtained by clamping the "kata" to a brass rod fixed at one end to the central pillar of a Sherrington recording drum, and revolving it in a horizontal circle; (ii) other observations were made in the respiration chamber, which is a closed room of $10{\cdot}5 \times 6{\cdot}5 \times 4{\cdot}5$ ft. capacity. The experiments by the whirling arm method at Hampstead were very similar to those carried out at Oxford. A screen of American cloth, 4 ft. high, was erected on the floor of a large room, so as to form an enclosure 4 ft. square, the top being open. The door and windows of the room were closed, so that the air inside the enclosure was still. A transparent celluloid window was inserted all round the screen at the height of the "kata" stem, so that readings might be taken of the cooling, and the thermometer read.

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* 'Phil. Trans.,' B, vol. 207, pp. 183–220 (1916).

† 'Roy. Soc. Proc.,' B, vol. 90, pp. 438–447 (1919).

In the whirling arm method the actual velocity, v, of the "kata" is given by

$$v = \frac{2\pi r n}{t},$$

where r is the radial distance of the "kata" from the axis of revolution, n the number of revolutions in the time t (estimated by stop-watch). L. V. King* and others have shown, however, that this is not the true velocity relative to the air, a certain "swirl" being set up by the revolution of the arm. This was determined and allowed for in our observations. The procedure by which this swirl was allowed for will be described later.

The Dry "Kata"—Wind Velocity and Temperature.

As shown in the previous papers, sources of radiant heat being absent, the rate at which the "kata" cools in air depends almost entirely upon the wind velocity and air temperature. It is shown that the heat loss per second per square centimetre of bulb surface is obtained by dividing a "factor," determined for each instrument by the time of cooling, expressed in seconds, which the alcohol meniscus takes to fall from 100° to 95° F., the unit of heat being a millicalorie (1000 millicalories = 1 grm. calorie). This is known as the "cooling power," H, and it was found that a connection between the variables is given by an expression of the form

$$H = (a + b\sqrt{v})\,\theta$$

where v is the wind velocity and θ the difference between the mean temperature of the range of cooling, viz., 36·5° C. (97·7° F.), and the air temperature in degrees Centigrade, whilst a and b are numerical constants.

To obtain the constants a and b, a number of observations of the cooling power were taken, the temperature and wind velocity being accurately determined at the same time. The results are shown graphically in figs. 1 and 2.

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* L. V. King, 'Phil. Trans.,' A, vol. 214, p. 373 (1914).

Each point in these graphs is the mean value obtained from three to seven observations of cooling, taken consecutively. Where points coincide the observations were taken at different times. Several kata-thermometers were used, the factors in each case being verified. The high velocity observations were taken in the wind tunnels and the low velocity ones by the whirling arm method. Unfortunately, owing to experimental difficulties, it was not found possible to make these two methods overlap, the lowest wind tunnel velocity available being 3·05 metres per second, and the highest whirling arm velocity for the dry "kata" 1·77 metres per second.

No equation of the above form will apply for all velocities over the range tested, but from the value $\sqrt{v} = 1$ to $\sqrt{v} = 4{\cdot}2$, that is, between the velocities 1 metre per second and 17 metres per second, a curve may be drawn to fit the points as shown, the equation of which is

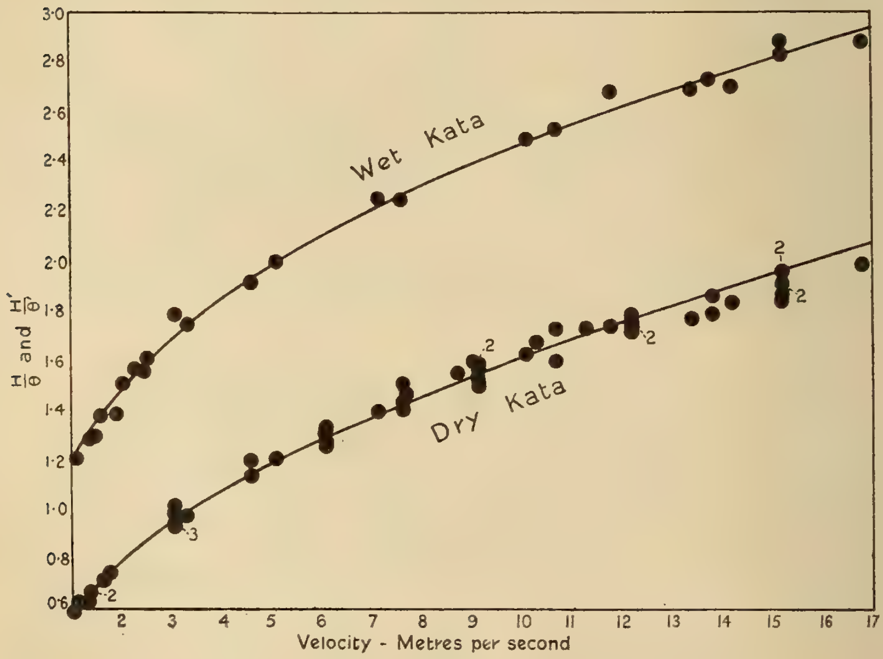

FIG. 1.

$$H = (0{\cdot}13 + 0{\cdot}47\sqrt{v}) \,.\, \theta. \qquad \text{(i)}$$

It should be noted that the above limits include observations in air currents obtained by both methods. Below 1 metre per second velocity another curve may be drawn, of which the equation is

$$H = (0{\cdot}20 + 0{\cdot}40\sqrt{v}) \,.\, \theta. \qquad \text{(ii)}$$

These two lines are shown with the experimental points as the lower curves in the diagrams.

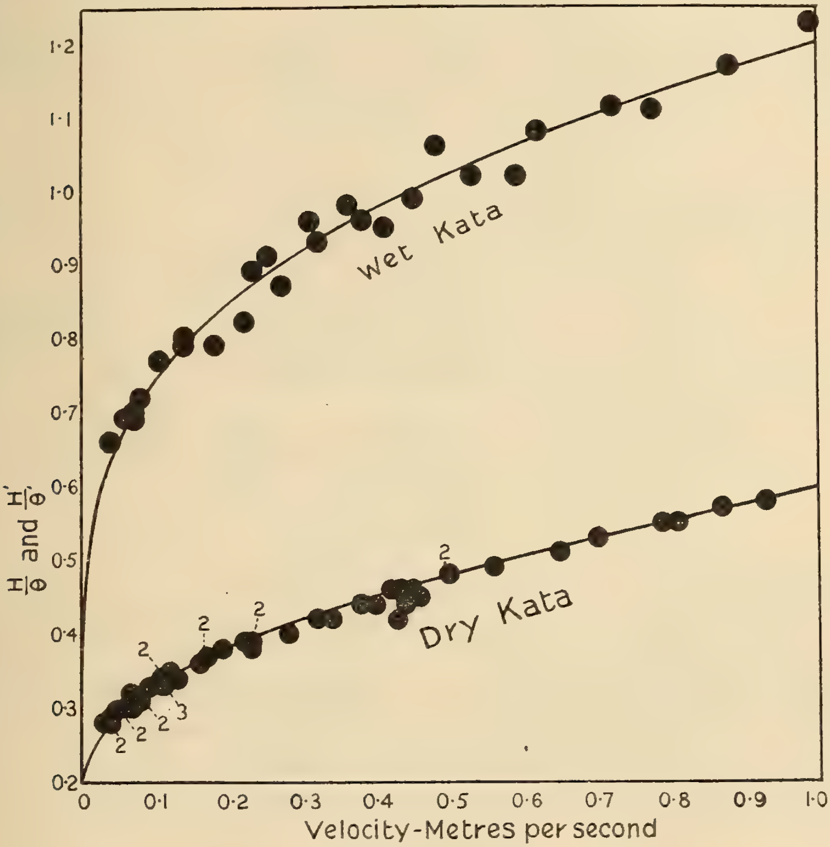

FIG. 2.

The Wet "Kata"—Wind Velocity, Temperature, and Evaporation.

Wet "kata" observations were made in a similar manner to the dry "kata" ones, except that the bulb of the instrument was covered with a muslin "finger," which remained moist during the time of cooling. In this case a velocity as high as 2·50 metres per second was obtained by the whirling arm method. With the wet "kata" heat is lost by the evaporation which takes place in addition to the heat lost by convection and radiation, as in the dry "kata," so that the wet cooling power, H′, is always greater than the dry cooling power, H. It is evident, then, that in addition to the temperature and wind velocities, some term must be introduced to take account of the humidity of the air. In the 'Phil. Trans.' paper the formula deduced from theory and experiment was of the form

$$H' = (a+b\sqrt{v})\,\theta+(c+d\sqrt{v})\,(F-f)^{4/3},$$

where H′ was the heat lost per second per square centimetre from the wet "kata," v the wind velocity, F the maximum vapour pressure at 36·5° C., f the actual vapour pressure of the air at the time of the observation, θ equal to the difference between 36·5° C. and the air temperature, and a, b, c, and d numerical constants. This was afterwards modified to the form

$$H' = (a' + b' \sqrt{v})\,\theta + (c' + d'\,v^{0\cdot 3})\,(F - f)^{4/3},$$

which was given in the later paper.

Our new investigations give an equation of the form

$$H' = (a'' + b'' \sqrt{v})\,\theta + (c'' + d'' \sqrt[3]{v})\,(F - f)^{4/3},$$

but, just as for the dry "kata," two equations are found to be necessary, one for velocities greater than 1 metre per second and one for velocities less than this. These equations are

$$H' = (0\cdot 13 + 0\cdot 47 \sqrt{v})\,\theta + (0\cdot 035 + 0\cdot 098 \sqrt[3]{v})\,(F - f)^{4/3} \qquad \text{(iii)}$$

for velocities greater than one metre per second, and

$$H' = (0\cdot 20 + 0\cdot 40 \sqrt{v})\,\theta + (0\cdot 060 + 0\cdot 073 \sqrt[3]{v})\,(F - f)^{4/3} \qquad \text{(iv)}$$

for velocities less than 1 metre per second.

In each case the first term on the right-hand side of the equation represents the dry "kata" heat loss.

These expressions are cumbersome for practical use, so that when the question was re-investigated we proposed to try if a formula in which the humidity was represented by the wet bulb temperature would give satisfactory results; that is to say, a formula of the form

$$H' = (a + b\,v^{x})\,\theta',$$

where θ' is the difference between 36·5° C. (97·7° F.) and the *wet* bulb temperature, t', a and b being numerical constants, and x some power to which the velocity, v, had to be raised.

When our experimental values of H'/θ' were plotted against the wind velocity values, as shown in the upper curves of figs. 1 and 2, it appeared that such a relation existed between the variables, and that, considering the whole range of velocities from zero up to 17 metres per second, the value $x = 1/3$ gave satisfactory results.

We may, therefore, write an empirical wet "kata" formula in the form

$$H' = (a + b \sqrt[3]{v})\,\theta',$$

where H′ is the heat loss per second per square centimetre, v the wind velocity, and θ' the difference between 36·5° C. (97·7° F.) and the wet bulb temperature, t'. As before, two equations are necessary to cover the whole range. The constants a and b, when v is in metres per second, H′ in millicalories per second, and θ' in degrees Centigrade, are given by the following two equations:—

$$H' = (0{\cdot}10+1{\cdot}10\sqrt[3]{v})\,\theta' \text{ for velocities greater than 1 metre per second,} \quad \text{(iii}a\text{)}$$

$$H' = (0{\cdot}35+0{\cdot}85\sqrt[3]{v})\,\theta' \text{ for velocities less than 1 metre per second.} \quad \text{(iv}a\text{)}$$

Our results with the wet "kata" in winds are only over a limited range of humidities (the wet bulb temperature varying from 9·5° to 19·8° C.), owing to the difficulty of controlling the humidity in the large rooms in which the wind tunnels are situated. In the case of still air, however, it is possible to obtain more varied conditions, and considering the still air wet "kata" formula

$$H' = 0{\cdot}27\theta+0{\cdot}085\,(F-f)^{4/3}, \qquad \text{(v)}$$

given in the original paper, it seems probable that for very dry or very moist air, equations (iii) and (iv) are more correct than (iii$a$) and (iv$a$). It is not possible to find an equation for still air of the form

$$H' = a\theta'.$$

This can be seen at once by putting in fixed values of the wet bulb temperature in (v) and varying the humidity. In this way it is found that a varies from 0·5 to 0·7 as the temperature and humidity vary from 10° C. and 10 per cent. relative humidity to 20° C. and 100 per cent. relative humidity. For any given wet bulb temperature the value of a increases with the humidity, the increase being much more rapid at low relative humidities.

With respect to the wind velocity index, when considering evaporation, we find that observers have come to many different conclusions on the matter. John Dalton gives the value as unity. A number of late investigators found it to be 0·5.* Recent observations made by Bigelow,† in which he determined the rate of evaporation of water from pans 2 to 6 ft. in diameter in wind of various known velocities, gave results agreeing with Dalton's. It may be pointed out that for certain ranges of wind velocity, *e.g.*, between the velocities of 0·3 and 2·5 metres per second, our results hold for a value of 1 for this index, but many more than two equations would be necessary to cover the whole range of our experimental work with this value, while for the index of 1/3 very close agreement is obtained by the use of two equations of the same form. The velocities recorded by Bigelow were mainly low. It can hardly be expected that the conditions of evaporation from large vessels and from such instruments as the "kata," where the evaporation surface is vertical and the water only a thin film, should be identical.

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* *Cf.* Hann, 'Lehrbuch der Meteorologie,' Ed. 3, 1915.

† Bigelow, 'Monthly Weather Review,' 1910, p. 307.

It will be noticed that the rate of cooling, both of the dry "kata" and the wet "kata," begins to take an abnormal course at the same point, viz., an air velocity of 1 metre per second. There can be little doubt that the abnormalities are due to convection currents. Currents of warm air, heated by the cooling "kata," tend to rise vertically from the sides of the bulb, and this air considerably impedes the rate of cooling of the "kata" in still air. It can have little or no effect in the presence of horizontal air currents of fair velocity, as it will be swept aside, but air currents of low velocity are not sufficiently powerful to effect this completely, and in consequence the rate of cooling is retarded.

Determination of Swirl.

It has already been mentioned that in the whirling arm experiments account must be taken of the swirl produced; that is to say, the true velocity of the "kata" relative to the air is less than the actual velocity, because the air is carried round with the revolving arm to a certain extent. A method similar to that described by L. V. King* was adopted to determine the amount of swirl. In the case of the Oxford experiments, a "kata" was fixed at one end of a horizontal arm and it was revolved at the rate of 1, 2, or 3 metres per second, and its bulb passed close to the bulb of another "kata," which was fixed in a stationary position. Cooling observations of the stationary "kata" were made while the moving one revolved at room temperature. When the centres of the bulbs were 2 cm. apart there was only 2 mm. space between the bulbs.

From the series of data obtained we were able to calculate what the H/θ values would have been had it been physically possible for the bulb of the stationary "kata" to occupy the place through which the bulb of the moving "kata" passed in its revolutions. From them it has been possible to calculate the velocity of swirl of air against the stationary "kata" bulb by using an approximate formula already determined from observations taken without allowance being made for swirl. These swirl velocities vary from 6·5 to 7·96 per cent. of the velocity of the revolving "kata," but they are all subject to a certain deduction. Now the velocity of swirl found by King when using a wire was 6 per cent.; therefore, assuming that this value holds likewise with the "kata"—the assumption being warranted by the above experimental results—then it follows that the values must be reduced by 6 per cent. This correction has been applied to all the Oxford results.

Similar experiments at Hampstead gave a value for the swirl of about 9 per cent., which was deducted from the calculated velocities before recording.

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* L. V. King, 'Phil. Trans.,' A, vol. 214, p. 373 (1914).

The Kata-thermometer in Practical Use as an Anemometer.

To test the accuracy of the wind formulæ for practical purposes, series of experiments have been carried out at Kew Observatory, where observations were taken by kind permission of Dr. Chree; at High Beech, Loughton; at Walberswick, by Mr. P. F. Alexander; and at Eskdalemuir, by Mr. P. N. Shelton, under the direction of Mr. L. F. Richardson. The results are shown in the Table. It will be seen that in all cases the wind as determined by the "kata" agrees well with that given by other anemometers.

Place of observation.	H.	$\theta = (36{\cdot}5 - t)$.	Velocity "kata."	Cup anemometer.	Kew anemometer.	Eskdalemuir air meter.	Time of observation. mins.
Kew	24·2	17·5	7·1	6·4	6·0	—	8
	21·5	18·0	5·1	5·7	6·0	—	3
	21·9	18·0	5·4 } 5·8	5·5 } 5·7	6·3 } 5·9	—	5
	22·9	17·9	6·0	5·5	4·9	—	7
	21·9	17·9	5·6	5·7	6·0	—	7
High Beech	25·1	22·0	4·7	4·9	—	—	5
	27·0	22·0	5·5	5·7	—	—	10
	25·5	22·2	4·7 } 5·4	5·5 } 5·6	—	—	10
	27·3	21·5	5·9	5·4	—	—	6
	28·6	22·0	6·2	6·5	—	—	6
Walberswick	35·5	33·2	4·0	4·0			
	35·5	33·2	4·0	4·4			
	48·0	37·3	6·1	5·3			
	33·1	37·6	2·5	2·8			
	45·1	37·5	5·2	5·0			
	55·5	37·6	8·3	7·5			
	40·4	37·1	4·2	4·6			
	32·5	34·6	3·0	3·0			
	29·0	28·2	3·7	4·1			
	32·0	30·4	3·8	4·2			
	29·4	29·6	3·3	3·5			
Eskdalemuir	23·2	33·6	1·3	—	—	2·1	
	34·6	33·8	3·6	—	—	2·9	
	27·7	33·1	2·2	—	—	2·4	
	24·2	33·1	1·6	—	—	1·7	
	22·5	30·3	1·7	—	—	0·7	
	40·1	35·8	4·4	—	—	4·7	
	26·8	32·8	2·2	—	—	2·3	
	35·8	29·5	5·3	—	—	5·2	
	31·5	33·2	3·0	—	—	2·5	
	26·7	32·7	2·2	—	—	2·2	
	36·8	30·6	5·2	—	—	3·5	
	28·0	31·6	2·6	—	—	2·4	

Summary.

From the above considerations then we consider that we are justified in saying that the "kata" is an instrument which shows a definite relation between the heat loss and the surrounding conditions, and that this relation may be expressed in the form

$$H = (a + b\sqrt{v})\,\theta$$

for the dry "kata," where H is the heat loss per second per square centimetre of "kata" bulb surface, θ being the difference between 36·5° C. and the dry-bulb temperature, and v the wind velocity in metres per second. The equation takes the following numerical forms for air velocities above and below 1 metre per second:

$$H = (0{\cdot}13 + 0{\cdot}47\sqrt{v})\,\theta \quad \text{(ii)} \qquad \text{and} \qquad H = (0{\cdot}20 + 0{\cdot}40\sqrt{v})\,\theta. \quad \text{(iii)}$$

The wet "kata" formulæ are more complex (see p. 201), but for ordinary atmospheric temperatures and humidities an approximation is yielded by the formula

$$H' = (a' + b'\sqrt[3]{v})\,\theta',$$

where H′ is the heat loss per second per square centimetre of wet "kata" bulb surface, and θ' is the difference between 36·5° C. and the wet bulb temperature.

This equation takes the following numerical forms for air velocities above and below 1 metre per second,

$$H' = (0{\cdot}10 + 1{\cdot}10\sqrt[3]{v})\,\theta' \quad \text{(iv)} \qquad \text{and} \qquad H' = (0{\cdot}35 + 0{\cdot}85\sqrt[3]{v})\,\theta'. \quad \text{(v)}$$

(It should be noted that the above equations are not true when v is less than 0·04 metre.) By means of equations (ii) and (iii) the "kata" may be used as an anemometer, both for estimating wind velocities out of doors, or the velocity of air currents indoors for purposes of ventilation. For the latter purpose it has a special value, for it estimates cooling effects of air currents whether unidirectional or eddying.

On the Heating and Cooling of the Body by Local Application of Heat and Cold.

By Leonard Hill, M.B., F.R.S., D. Hargood-Ash, B.Sc., and J. Argyll Campbell, M.D.

(Received January 7, 1922.)

(From the National Institute for Medical Research, Hampstead.)

The object of this enquiry is to find out how much heat can be gained, or cold lost from the body, by the local cooling or warming of a small part, *e.g.*, by cooling the hands in a stream of cold water, warming the feet in a hot foot-bath, or by a foot-warmer. In order to secure the beneficial effect of open windows, the breathing of cool air of low-vapour tension, and stimulation of body metabolism by such air ventilating the clothed and naked parts of the skin, the general heating of rooms by hot-water coils might be replaced by small heaters kept a few degrees above body temperature and locally applied to each individual, and each under the individual's control. Electric heaters have been used by aeroplanists placed beneath their outer garments.

One of us (1) recently published results showing that heating or cooling the hands can effectively heat or cool the whole body. We record further experiments of a like nature.

In some of these experiments, in which the hands were placed in cold water, we estimated the respiratory exchange by the Douglas-Haldane method of indirect calorimetry. We obtained in most cases a small rise in body metabolism after immersion of both hands in cold water at 15° C. for about 30 minutes. The rise in metabolism was too small to be termed definite. The amount of heat lost from the hands to the water was evidently replaced by cutting down heat lost from other parts of the body. The actual amount of heat lost from the two hands in 30 minutes was, on an average, 20 kilo-calories. A greater loss of heat is therefore necessary before metabolism is affected.

The temperature of the skin over the median vein at its bifurcation on the front of the elbow was recorded by means of a flat-coiled thermometer insulated from the air. The temperature of this portion of skin fell several degrees in the above experiments.

Macleod (2) and others have applied heat and cold to the surface of the shaved body of rabbits to study the changes of temperature in underlying tissues and in various organs—muscle, liver, kidney and brain. They used thermo-electric couples mounted in hypodermic needles. They showed that the changes produced vary with vascularity in different tissues. Heat applied over the liver had little effect on the liver, compared with that on muscle, of heat applied over the same. Applied over the lung both heat and cold had little effect; over the brain there was a prompt change of brain temperature but little effect on general body temperature.

In our experiments to determine the amount of heat lost by the hand to the water, the hand was placed in a tin can containing a known volume of water; a similar can with a similar volume of water and at approximately the same temperature was placed beside the former to act as a control. When the temperature of the skin over the median vein was found to be constant, the hand was immersed in the water as far as the wrist joint. The temperatures of the water-bath and the control were read immediately before the immersion of the hand, and readings were then taken of the skin temperature at the elbow, and of the bath and control temperatures, every minute.

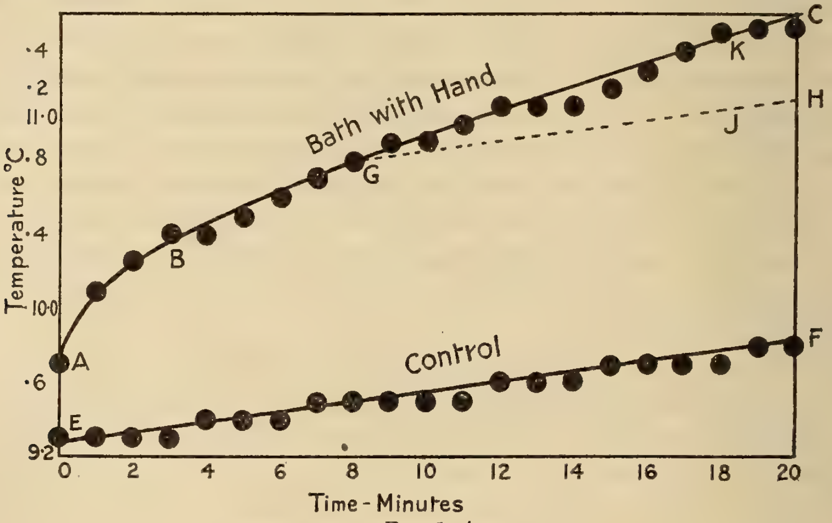


Fig. 1, A.

To determine the heat given to the water, the temperature readings of the bath and control were plotted. Fig. 1, A shows a typical example. The initial temperature of the bath was 9·7° C. and of the control 9·3° C. The bath curve, ABC, shows that for the first 3 minutes the rate of rise of temperature was rapid compared with the rate afterwards. From the eighth to the twentieth minute the rate of rise of temperature may be taken as constant. The curve, EF, shows the rise of temperature of the control bath; it will be seen that this rate of rise was constant throughout the experiment. GH is drawn parallel to EF, therefore the difference between the lines GC and GH gives the rise of temperature due to the immersion of the hand. At the point JK, 10 minutes from point G, the difference is 0·38° C., therefore the rise for 1 minute was 0·038° C. The water in the can was 6 litres, therefore the rate of heat loss to the water per minute was 0·228 kilo-calories during this time.

To determine the total heat loss from the hand during the 20 minutes' immersion; the total rise of temperature of the bath was 11·6° C.−9·7° C. = 1·9° C., and that of the control 9·8° C.−9·3° C. = 0·5° C.; therefore the total rise due to the hand was 1·9° C.−0·5° C., = 1·4° C., and the total heat loss from the hand 1·4 × 6 = 8·4 kilo-calories.

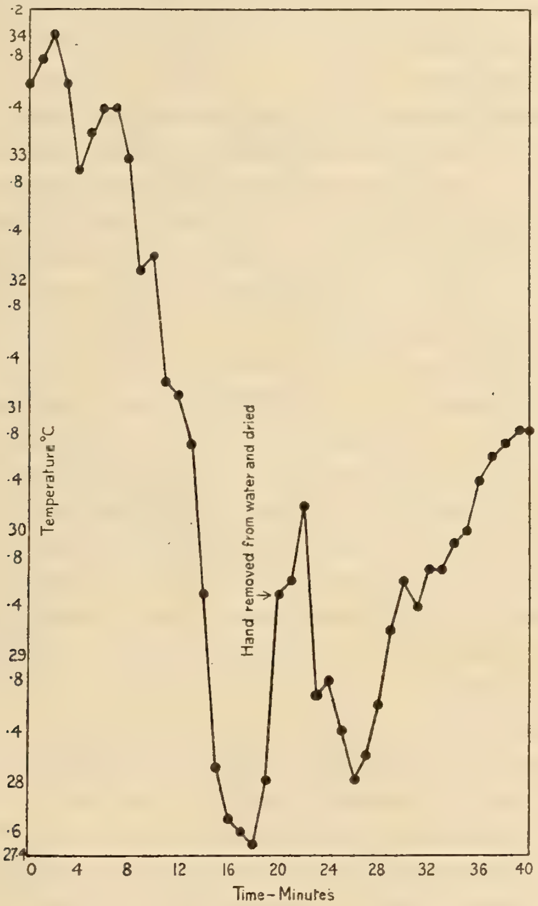


FIG. 1, B.

Fig. 1, B, shows the changes in temperature of the skin over the median vein for the 20 minutes' immersion and the succeeding 20 minutes. The temperature of this portion of skin was quite different from that of the skin around it and nearer the hand, showing that the temperature of the blood in the vein was the controlling factor, and not conduction by the skin from tissues affected by the changes in the hand.

Pain in the hand was intense during the first few minutes if the temperature of the water was below 14° C. This pain may probably be due, in part, to the extreme contraction of the tissues, and disappeared probably because the fluids escaped up the arm from the contracted tissues in the hand, thus relieving pressure. In our metabolism experiments already referred to, we used water warmer than 14° C., to avoid the influence of pain. Pain is known to raise metabolism.

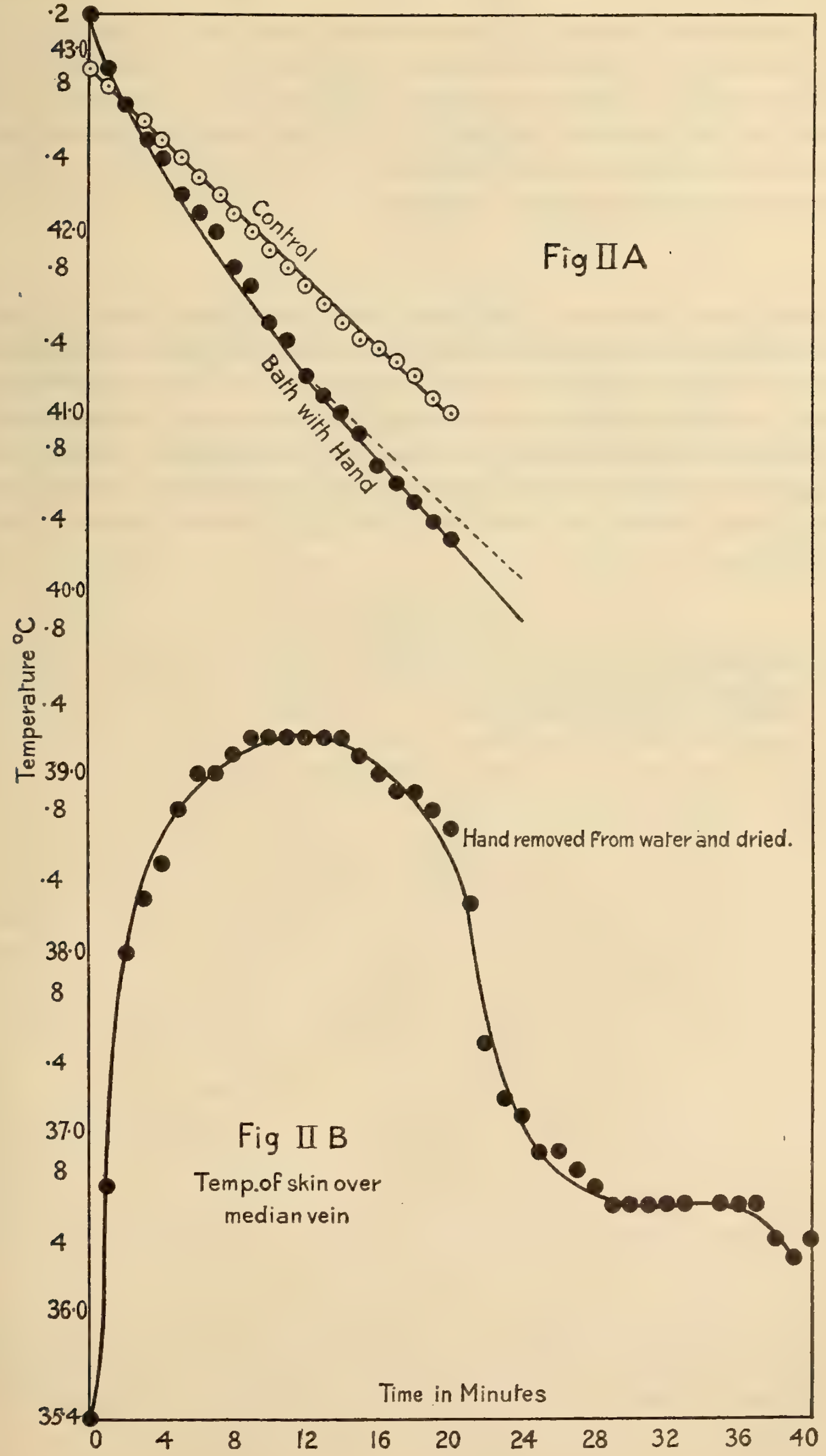
In our numerous experiments, with the hand in cold water, we were unable to obtain from day to day constant results for a given range of temperature, probably because so many bodily conditions, *e.g.*, vascular, are variable.

The double fig. 2 gives the results of an experiment in which the hand was placed in hot water (43·2° C.), fig. 2, A, recording the change in temperature of bath and control, and fig. 2, B, recording the changes in temperature of the skin over the median vein at the point of the elbow. In this experiment the heat gained by the hand in 20 minutes was 6 kilo-calories, and the rate of gain, after this had become constant, 0·120 kilo-calories per minute.

In these experiments with hot water the fall of temperature in the water of the bath was considerably greater during the first minute or so after the hand was immersed, and it was difficult to tell how long this effect, which was probably due to the heating up of the hand, lasted. If this effect was only temporary, and the heat gained depended only upon the temperature of the water, we should expect that different experiments would give curves parallel to each other over the same range of temperature. This, however, did not occur.

To obtain more certain information on this point it was decided to immerse the hand in hot water as before, but, instead of allowing the water to cool, to supply a constant amount of heat equal to that lost by the water, so that if the heat absorbed by the hand at a given temperature proved to be constant, the experiments could be repeated at different temperatures to obtain a curve. However, the heat absorbed by the hand at a given temperature was not found to be constant, similar variations being obtained as with cold water; varying bodily conditions were probably responsible.

We also carried out numerous experiments with the flat-coiled thermometer, when the hand as far as the wrist was heated by the summer sun's rays. The temperature of the skin over the median vein was increased several degrees after the hand had been exposed for a few minutes. In these cases the blood in the vein may have been heated in the hand by conduction


·2
43·0
·8
·4
42·0
·8
·4
41·0
·8
·4
40·0
·8
·4
39·0
·8
·4
38·0
8
·4
37·0
8
4
36·0
35·4
Temperature °C
Control
Bath with Hand
Fig II A
Hand removed from water and dried.
Fig II B
Temp. of skin over median vein
Time in Minutes
0
4
8
12
16
20
24
28
32
36
40

from the skin or by rays which had penetrated the skin and had been converted into heat in the blood, as pointed out by Sonne (3). He used thermoelectric couples to determine the effect of different rays from the sun and found that the visible rays penetrate the skin and locally heat up the blood. To this he attributes the value of the sun in heliotherapy. Dark heat rays heat up the surface of the skin much more.

Summary.

Experiments in which the hands were heated or cooled by water showed that the amount of heating or cooling was large, but not constant, for a given range of temperature. Some indication of the degree of heating or cooling was obtained from the temperature of the skin over the median vein at the elbow, the thermometer used being coiled and insulated from the air. Loss of 20 to 25 kilo-calories of heat from the hands in 30 minutes, *i.e.*, a loss almost equal to the basal metabolism, did not appreciably affect the body metabolism.

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(2) Macleod, Self, and Taylor, 'Lancet,' September 25, 1920, p. 645 ; Macleod and Taylor, *ibid.*, July 9, 1921, p. 70.
(3) Sonne, 'Acta Medica Scandinavica,' vol. 54, p. 394 (1921).

On the Oxidation Processes of the Echinoderm Egg during Fertilisation.

By C. SHEARER, F.R.S.

(Received November 15, 1921.)

I. INTRODUCTION.

The following paper is concerned with an investigation of the oxidation processes of the animal egg-cell during fertilisation. The subject has already received considerable attention and the problem has been approached from many different aspects. The first to attempt to measure in definitive quantitative manner the oxygen consumption of the egg on fertilisation, was Warburg (1) in 1908. He made use of the sea-urchin *Arbacia*, and estimated the amount of oxygen that had disappeared from the sea-water in which the eggs had remained for some time. The Winkler titration method was employed. He found that a quantity of eggs that gave a Kjeldahl determination of 28 mgrm. of egg nitrogen, which corresponds roughly to about 4 million eggs, 4–5 c.c. of oxygen was taken up in the first hour following fertilisation, while the same quantity of unfertilised eggs only consumed 0·5–0·7 c.mm. of oxygen in this time. The fertilised egg, therefore, took up six to seven times more oxygen than the unfertilised egg. Loeb had previously predicted, that the main function of the sperm in the process of fertilisation was that of setting up a series of oxidations on its entrance into the cytoplasm of the egg. Warburg's work was a remarkable confirmation, therefore, of Loeb's prediction. This first paper was followed up by a long series of papers which have added greatly to our knowledge of the oxidation processes taking place in the egg on fertilisation. In addition, our knowledge has also been greatly extended by the numerous papers of Loeb, and especially the papers of Loeb and Wasteneys (2) in which quantitative measurements were also carried out. In 1911 appeared the large paper of Meyerhof (3) in which the heat liberation was measured and correlated with the oxygen consumption. In all these papers the Winkler method was employed; there are, however, many drawbacks to the use of this method, and in the recent work of Warburg and Meyerhof it has been finally abandoned for the more convenient and accurate manometer. The great advantage of the manometer method lies in the fact that it can be used equally well for both oxygen and carbon dioxide determinations, and that continuous observations can be carried out minute by minute on the respiratory exchange of the material under investigation. Warburg (4) (1915), using this instrument, has recently reinvestigated the respiratory exchange in the egg of the sea-urchin *Strongylocentrotus* during the first 24 hours of development. He found that a quantity of unfertilised eggs that contained 20 mgrm. of egg nitrogen, which corresponds to about 3 million eggs, consumed in 20 minutes 10–14 c.mm. of oxygen at a temperature 23° C. and barometer 760 mm. Hg. The fertilised egg under the same conditions, 10 minutes after the addition of the sperm, consumed 60–84 c.mm. That is 10 minutes after fertilisation the oxygen consumption of the egg was six times that of the unfertilised egg, and that there was already a rise of 500 per cent. in the oxidation rate of the egg in this time. In the sixth hour the oxygen consumption was twelve times that of the unfertilised egg, at 12 hours it was sixteen times, while at 24 hours it was twenty-two times the amount of the unfertilised egg. As Warburg remarks, it is extraordinary that in one and the same cell substance, which receives no addition of fresh material from any external source, we should find, as the result of fertilisation in the course of 24 hours, a rise in its oxidation rate of something like 2000 per cent. On the whole the manometer method seemed to show that there was a much closer agreement between the increase in the respiratory quotient and the appearance of visible structure in the egg, than had been demonstrated in previous work where the Winkler titration method had been employed. In all instances the CO_2 output of the eggs followed closely the oxygen uptake, the respiratory quotient being in the neighbourhood of 0·9. The respiration of a single spermatozoön was found to be about 1500-2000 times less than that of the egg.

In the past season, working at Naples, I have been able to carry the investigation of the problem a step farther, by the use of a special type of the Barcroft differential manometer, in which it was possible to bring about the fertilisation of the eggs in the closed chamber of the apparatus, and so for the first time the measurement of the respiratory exchange during the period the sperm were actually making their way into the egg was rendered possible. The eggs and sperm of *Echinus microtuberculatus* were used.

II. METHODS.

In the previous experiments of Warburg (4) (1915), where the manometer has also been employed, readings were only obtained 10 minutes after the addition of the sperm. The following experiments will show that, by the end of this time, the most important part of the process of the fertilisation of the egg has already taken place. There is a great initial inrush of oxygen into the egg and a corresponding output of CO_2 within the first minute after the addition of the sperm to the eggs. It is clear that the spermatozoön sets up an instantaneous oxidation process in the egg, which is perhaps unparalleled in reactions of the animal cell for its sudden character.

In the type of manometer employed in the following experiments the instrument, when used for ordinary blood-gas measurements is designed to allow a small quantity of potassium ferricyanide, held in a small tube in the stopper of the manometer chamber, to run down and mix with the blood when the chamber itself is slightly rotated, so that a groove in the chamber wall comes to lie opposite the opening of the small tube in the chamber stopper. The instrument was readily adapted to the purpose of the present experiments by replacing the ferricyanide by a drop of fresh sperm. The eggs could then be fertilised within the closed chamber of the apparatus, when both eggs and apparatus were in complete equilibrium with the temperature of the water-bath in which the manometer chambers were submerged.

The calibration of the manometers for oxygen was carried out by the Hoffmann (5) method, and for CO_2 by the liberation in the chamber under calibration of a known volume of this gas from 2 c.c. of a Na_2CO_3 solution. All calibrations were carried out under the conditions holding for the following experiments. The temperature of the water of the thermostat in which the chambers of the manometer were submerged was kept constantly at 14·5° C. by means of a coil of piping inside the bath, through which cold water was kept circulating. This temperature is almost the same as that at which the eggs are normally fertilised in the sea. Moreover, it is convenient, as the solubility of CO_2 in sea-water at this temperature and standard barometric pressure is almost 1, so that the CO_2 will distribute itself evenly throughout both sea-water and the air space of the apparatus chambers, and no correction need be introduced for solubility of the CO_2 in the sea-water on this account.

Warburg (4), in his measurement of the CO_2, has taken elaborate means to determine the actual amount of CO_2 given off, by using bicarbonate-free sea-water and estimating the total alkali reserve of his eggs; it is probable that the use of bicarbonate-free sea-water in itself introduces conditions that render respiration far from normal.

In a number of determinations made on the solubility of CO_2 in sea-water at the pressures obtaining under the conditions holding in the chambers of the manometer in the following experiments, it was found this solubility could be neglected, as it was so small in each case as to be well within the error of the manometer reading.* It is only at tensions of 0·4 mm. pressure, or below this, that a great difference is made in the solubility of CO_2 in sea-water. Tensions of this order never obtained in the chambers of the manometer under the conditions of the following experiments. In the following experiments no special importance was attached to the carbon dioxide measurements beyond determining roughly the relation of the carbon dioxide output to the oxygen consumption. No trouble was taken, therefore, to determine the CO_2 output with any degree of accuracy. It is clear that it follows the oxygen consumption very closely, the respiratory quotient being always in the neighbourhood of 0·9.

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* The change of pressure in the chamber of the apparatus during an experiment was very small, being something of the order of 0·005 of an atmospheric pressure. The effect of this on the solubility of carbon dioxide in the sea-water of the chamber could safely be neglected.

To absorb the carbon dioxide in the oxygen manometer, a drop of KOH was placed in the small cup in the bottom of the chamber stopper, and this was renewed after each experiment.

The manometers were made to clamp on a mechanical shaker, such as that used when the instrument is employed for making blood-gas determinations. The motion of this shaker had to be slowed down very considerably into a very gentle to-and-fro motion, or otherwise considerable injury resulted to the egg-membrane. Any injury to the egg-membrane always results in an immediate and abnormal increase of the oxygen consumption of the egg. It was also found that the conical pointed type of chamber with which the instrument is furnished for blood-gas work was highly unsuitable for egg work. The eggs tend to crowd down in the narrow end of the bottle and there clump together, and fertilise very badly. The conical chamber was therefore replaced in the following experiments by a more or less spherical-ended pattern. In making a determination, invariably 2 c.c. of eggs in sea-water were placed in one chamber, while the same quantity of plain sea-water was placed in the control chamber. The chambers were then attached to the manometer and half submerged in the water of the thermostat; a drop of very dilute fresh sperm was then introduced by a fine pipette into the tube in the stopper of the chamber containing the eggs, through a small opening in the top of the stopper. When the glass plug closing this opening had been replaced, the chambers of the manometer could be completely *submerged in the water of the thermostat with the stop-cocks open. It was left at least* 20 *minutes with the shaker working, to come into complete equilibrium with the temperature of the water of the bath.*

If the eggs are not properly cooled to the bath temperature, or if the sperm, when run down on the eggs, are at a different temperature from the eggs, faulty manometer readings will be obtained. To avoid any such errors in the present experiments, great care was always taken to have the eggs, sperm, and manometer, all at exactly the same temperature as the water of the thermostat tank before commencing an experiment. The eggs and sperm, after being washed several times in clean sea-water, were placed in large open-mouthed bottles suspended in the water of the thermostat. They were taken from these bottles as required for an experiment and were therefore always at the same temperature as the bath water. The pipettes used to measure out the eggs and sperm were always kept in separate bottles of sea-water when not in actual use, these bottles being also suspended in the water of the bath. The manometers, finally, were always kept in position on the thermostat when not in use, so that when required for an experiment they were already thoroughly cooled to the temperature of the bath. As already mentioned, in addition to these precautions 20 minutes was always allowed after the eggs and sperm were placed in the manometer chamber before the stop-cocks were closed and the first reading taken. It was found from preliminary experiments in which plain sea-water was used instead of eggs and sperm, that when these conditions were observed in performing an experiment no cooling effect could be produced, and that, under these conditions, the first reading on the manometer was perfectly accurate and satisfactory. In revolving the chambers on the manometers the stop-cocks of the apparatus were opened and immediately closed a moment later. It was possible that, in the operation of revolving the chambers, they might be slightly loosened on their respective stoppers and so the oil drawn round, and a faulty reading obtained in this way. This was avoided if the stop-cocks of the instrument were open while the chambers were revolved. The chambers of my manometers were so accurately ground on to their respective instruments that I was unable, as a matter of fact, to produce any such error experimentally when I turned the chambers while the instruments were closed. An error of this kind could only be produced by actually forcing the chambers completely loose from their manometers. Observing the foregoing precautions then, at the end of 20 minutes the hands of the manometer stop-cocks could be closed and readings commenced on the unfertilised eggs. The same eggs could then be fertilised by turning the chambers and allowing the sperm to run down on the eggs. There was a small error in this reading due to the presence of the drop of sperm in the small tube in the manometer stopper, but care was always taken to use very dilute sperm so that this error could be neglected. To rotate the chambers, when they were attached to their instruments and the manometers themselves in position submerged in the water of the thermostat, wooden handles were wired on the necks of each chamber. These were long enough to project well above the surface of the water, so that the warm hand or fingers never came in close contact or touched the manometer chamber when they were rotated. Separate manometers were used for the oxygen and CO_2 measurements, the output of CO_2

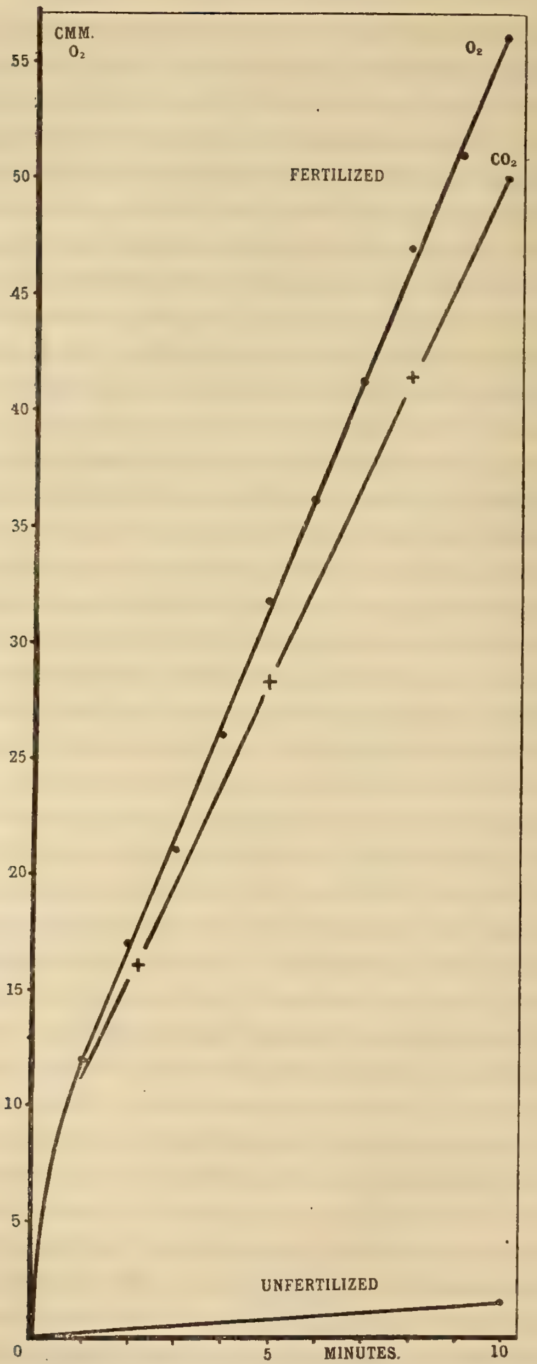

FIG. 1.—Graph showing the amount of oxygen taken up and the carbon dioxide given off during the fertilisation of half a million eggs (4·06 mgrm. of egg N) of *E. microtuberculatus*. The lower line shows the oxygen consumption of the same egg before fertilisation. Broken curve carbon dioxide output of eggs. Respiratory quotient 0·92. The quantities of oxygen and CO_2 are given in cubic millimetres plotted against time in minutes.

being arrived at by deducting the readings of the CO_2 manometer from the oxygen apparatus.

It was inevitable that a considerable difference in the amount of oxygen consumption on fertilisation should have been observed between different batches of eggs and sperm from different females and males. In some individuals the generative products were naturally more ripe and in better condition than in others. As the eggs and sperm for experiments of this kind have to be cut out of the animals and not laid by the animals in the normal manner in the sea, there is always some uncertainty as to their being perfectly mature.

The eggs can only be roughly tested by adding a little sperm to a few eggs in a watch crystal and seeing how evenly and quickly fertilisation takes place. As Warburg (4) remarks, it is obviously necessary in determinations of this kind that fertilisation should take place in all the eggs at the same moment, and that the rate of progress of this process, once it is set up in the eggs, should be the same in all the eggs. These conditions are not always successfully hit off in an experiment. In a certain small number of experiments out of some 200 to 300 performed, these conditions were probably as favourable as the experimental conditions would allow, and in these the readings were remarkably similar. In the following section the figures of three typical experiments of this kind are given. A graph of the readings is shown in figs. 1, 2, and 3, where the oxygen consumption in cubic millimetres is given plotted against the time in minutes.

III. EXPERIMENTS.

Unfertilised Eggs. (20 mgrm. egg N.)

The oxygen consumption of the unfertilised egg is so low that it is quite unmeasureable with any degree of accuracy, unless a much larger number of eggs is used than when a determination is made on fertilised eggs in the ordinary way. Instead of using one to half a million eggs, 4 to 5 millions (20 mgrm. egg nitrogen) were used in arriving at the correct estimation of the respiration of the unfertilised eggs.

The average (15 determinations) amount of oxygen taken up by 4 to 5 million unfertilised eggs, in 10 minutes, under the conditions of the experiments was 7 c.mm. oxygen; at this rate, half a million eggs (4 mgrm. of egg nitrogen) would consume 1·5 c.mm. in this time.

Fertilised Eggs.

Oxygen Manometer. (4·06 mgrm. egg N.)

1st	minute after addition	of sperm	12 c.mm.	oxygen	consumed.
2nd	„	„	17·0 c.mm.	oxygen	consumed.
3rd	„	„	20·0	„	„
4th	„	„	26·0	„	„
5th	„	„	32·2	„	„
6th	„	„	36·0	„	„
7th	„	„	41·4	„	„
8th	„	„	46·8	„	„
9th	„	„	50·0	„	„
10th	„	„	56·0	„	„

Carbon Dioxide Manometer. (4·08 mgrm. egg N.)

At end of	2	minutes after	addition of	sperm difference was	2	c.mm.
„	5	„	„	„	4	„
„	8	„	„	„	5	„
„	10	„	„	„	6	„

At the end of the experiment the manometer chambers were opened, when it was found that the eggs in both chambers had all formed normal fertilisation membranes and consequently all formed regular two-cell segmentation stages. A Kjeldahl determination on the eggs showed that in the case of the eggs in the oxygen chamber 4·06 mgrm. of egg nitrogen were present, and in the case of the CO_2 chamber 4·08 mgrm. of egg nitrogen were present.

It will be seen from the foregoing Tables and graph, fig. 1, that on addition of the sperm to the eggs there is an immediate consumption of oxygen. In the course of the first minute the uptake of oxygen is many times that of the same eggs one minute before the addition of the sperm, and more is usually taken up in the first minute than is taken up in the second and third minutes after the addition of the sperm taken together.

In all instances the CO_2 output of the eggs follows the oxygen uptake very closely, the respiratory quotient being in the neighbourhood of 0·92.

In fig. 2 is shown a graph of another experiment similar to the former, in which half the quantity of eggs were employed (2·08 against 4·06 mgrm. egg nitrogen). It will be seen both curves are similar, the later being half the value of the former.

At standard barometric pressure, and temperature of 14·5° C., 4·06 mgrm. of egg nitrogen (half a million eggs) which before fertilisation consumed

1·5 c.mm. of oxygen in 10 minutes, after fertilisation consumed 56 c.mm. in this time; there was thus an increase in the respiratory quotient of the eggs in 10 minutes after fertilisation of something like 37 times that of the unfertilised condition. If we consider the increase taking place at the end of the first minute after the addition of the sperm to the eggs, we get

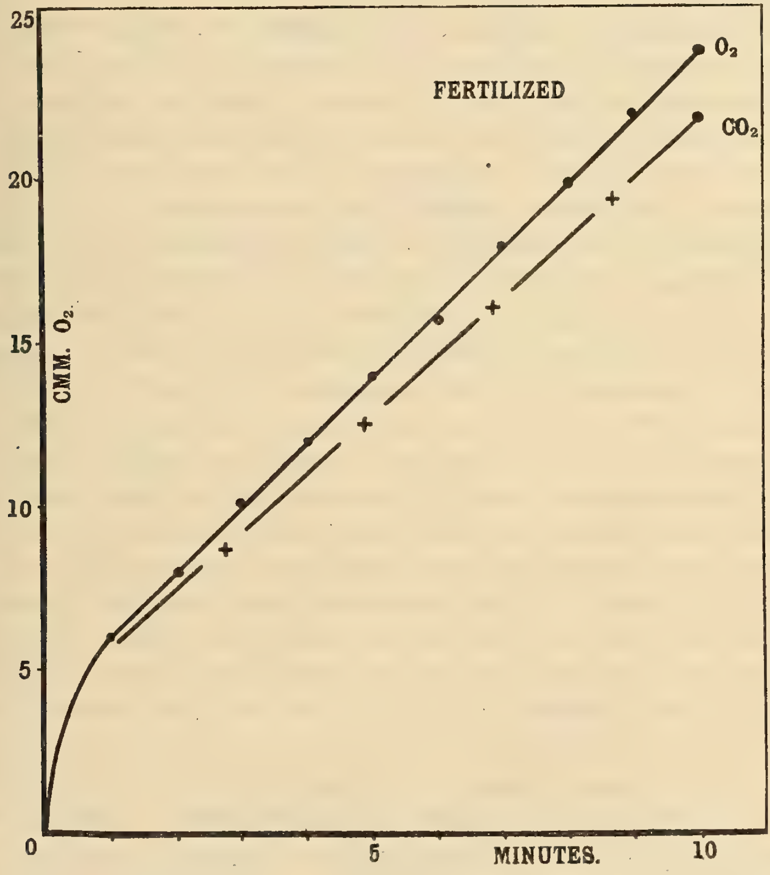

Fig. 2.—Graph showing the amount of oxygen taken up and the carbon dioxide given off during the fertilisation of a quarter of a million (2·08 mgrm. egg N.) eggs of *E. microtuberculatus*. It will be seen that this graph is similar in all respects with that shown in fig. 1, except that it is half the size of the former, half the quantity of eggs being used.

even more striking figures. The oxygen consumption of the unfertilised egg is, we have seen (15 determinations), about 1·5 c.mm. oxygen for 4 mgrm. of egg nitrogen in 10 minutes, and in the case of the present experiment a reading of this value was actually obtained on the unfertilised egg. If we divide this figure by 10 we arrive at the value of the oxygen consumption for 1 minute = 0·15 c.mm. The same eggs fertilised consumed in the first minute after the addition of the sperm 12 c.mm. of oxygen. Thus, the addition of the sperm to the eggs causes, within the space of 1 minute, an increase in their oxygen consumption of something like 80 times that observed on the same eggs 1 minute previous to the addition of the sperm.

The examination of sections of fixed material of the eggs of *E. microtuberculatus* during different stages of the process of fertilisation shows that the sperm take at least 10 to 15 minutes to enter the cytoplasm of the egg. In material fixed within 2 minutes of the sperm being added to the eggs, the sperm are seen only attached to the external surface of the egg membrane. They have not penetrated the membrane itself.

This initial oxygen consumption of the egg immediately on fertilisation must be brought about by the first contact of the sperm with the external surface of the egg membrane. We arrive then at the remarkable conclusion that contact of the spermatozoön with the external surface of the egg is capable of increasing its oxygen consumption in 1 minute by something more than 8,000 per cent. In fact, the total oxygen consumption of the eggs shown in figs. 1 and 2, represents some change brought about in the egg by the spermatozoön before it has entered the egg, and before it has formed a male pronucleus in the egg cytoplasm. It is moreover clear, that when the fusion of the male and female pronucleus takes place at a later stage of the process, it is not accompanied by any fresh rise in the oxygen consumption of the ovum, but instead, a slight drop in the curve is often observed about this time (see fig. 3). The nuclear features of syngamy therefore, seem connected in no direct way with the oxidations taking place in the ovum during fertilisation.

In fig. 3 is shown a graph of an experiment which illustrates these points. It represents the amount of oxygen taken up and the carbon dioxide given off during the fertilisation of a quantity of eggs that contained 2·8 mgrm. of egg nitrogen. The curve extends over a period of 1 hour after the sperm were added to the eggs, and so to a time when the first segmentation division has been completed and the egg has attained the two-cell stage. The readings are in 2-minute intervals up to the end of 10 minutes, and after this at 5-minute intervals. At the end of 2 minutes 10·5 c.mm. of oxygen had been consumed, which compares favourably for this quantity of eggs, with the figures obtained in the previous experiments, the graphs of which are shown in figs. 1 and 2.

It will be seen that at the end of 15 minutes the curve begins to appreciably flatten, and this flattening increases at the 25th minute when the fusion of the pronuclei is taking place. At the 40th to 45th minute it begins to rise again and this synchronises with the first segmentation division. The steepest part of the curve is that in the first 10 minutes interval, which corresponds to the action of the sperm on the external surface of the egg membrane.

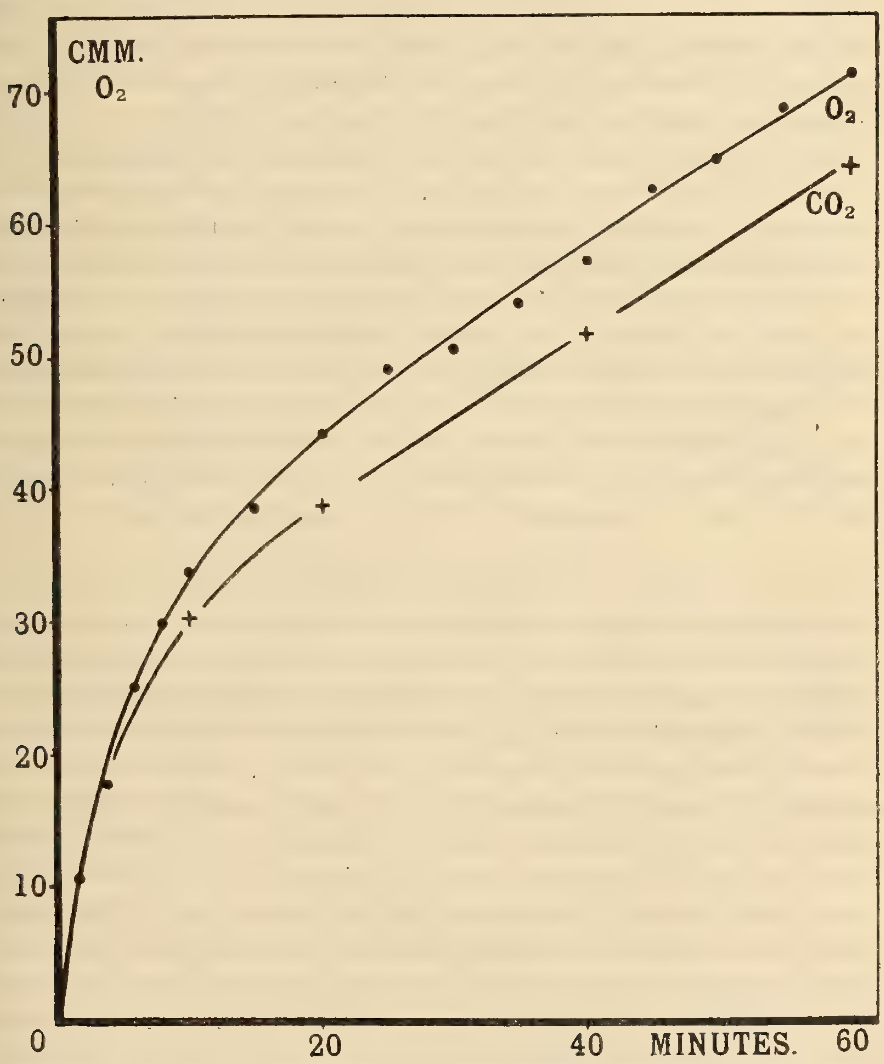

Fig. 3.—Graph showing the oxygen consumption and carbon dioxide output of a quantity of *E. microtuberculatus* eggs that contained 2·8 mgrm. of egg nitrogen during the course of the first hour following fertilisation. It will be seen that the steepest part of the curve is in the first 15 minutes, while the sperm are in relation with the egg membrane. At the 25th minute, when the male and female pronuclei are undergoing fusion, there is a slight flattening of the curve.

As the supply of free energy required by the developing ovum is mainly or entirely derived from its oxidation processes, it is surprising to find that during fertilisation, where a very large amount of energy is immediately rendered available, none of it is liberated as the result of the fusion of the egg pronuclei, a feature which heretofore has always been considered the most essential and important part of the whole process.

It is of interest in this connection to compare roughly the oxygen consumption of the ovum on fertilisation with that of the fixed tissues of the adult body. If we take liver tissue as one in which the metabolic rate is high, according to Barcroft and Shore (6), 1 grm. of fresh liver tissue from a well-fed cat consumes 0·024 c.c. of oxygen per minute, which is equivalent to 240 c.mm. in 10 minutes. If we take Schryver's (7) figures of the protein nitrogen-content of 1 grm. of well-fed cat's liver as 22·4 mgrm. nitrogen, we find the oxygen uptake of the ovum in the first 10 minutes of the fertilisation process, as compared with that of a similar quantity of liver protein in the same time, is in about the ratio of 13·8 to 10·7 in round figures. In the unfertilised egg this proportion is 0·37 to 10·7 for the same time.

IV. Discussion.

There are good reasons for believing, as the result of Loeb's (8) experiments on the fertilisation of the eggs of *Strongylocentrotus* with the sperm of *Asterias*, and Lillie's (9) description of the process of fertilisation in *Nereis*, that the entry of the spermatozoön into the egg consists of two distinct phases. Firstly, an external one, in which certain changes are brought about in the cortical substance of the egg the moment the sperm make contact with the external surface of the egg-membrane; this would seem to be correlated with the initial oxidation taking place in the egg, as described above for *E. microtuberculatus*. Secondly, the changes following the actual entry of the spermatozoön into the egg cytoplasm itself, which, as Lillie has shown in *Nereis*, only takes place some 30 minutes after the first phase of fertilisation, and in the sea-urchin follows some 10 to 15 minutes after the sperm are added to the eggs.

By centrifuging the eggs of *Nereis* before the sperm has actually penetrated the egg-membrane, Lillie was able to separate the jelly surrounding the egg and containing the spermatozoön from the egg itself. These eggs complete meosis, which has been initiated by the spermatozoön, but never segment. A typical segmentation nucleus is, however, formed, which breaks down leaving the chromosomes free in the egg cytoplasm; they split longitudinally in the normal manner, but never separate. No asters or miotic spindle appear in these eggs, as when the complete process of fertilisation is allowed to take place; and in the absence of these structures the process of cell division makes no further progress, and the chromosomes finally degenerate and break down. This experiment clearly proves that the sperm bring about profound alterations in the egg while still external to the egg-membrane. Loeb (8) has shown that when the eggs of *Strongylocentrotus* are fertilised with the sperm of *Asterias*, in hyper-alkaline sea-water, they only form fertilisation membranes: no actual segmentation takes place unless the eggs receive further treatment so that artificial parthenogenesis is induced.

Meyerhof and Warburg in many of their experiments have shown that any injury or cytolysis of the egg-membrane is invariably followed by a great increase in the oxygen consumption of these eggs. Meyerhof (10) found that this is usually accompanied by an increased liberation of heat. In eggs treated with weak solutions of NaCl, in which the normal condition of the cell-wall is destroyed in the absence of Ca and K ions, the rise of oxygen consumption was five times that of the same quantity of untreated eggs. The heat production was increased from 0·9 grm.-calories per hour to 3·4 grm.-calories per hour, after treatment with valerianic acid, by which artificial membrane formation had been induced.

A great many of Loeb's and Warburg's experiments point conclusively to the cortical layer of the egg and the egg-membrane as being the controlling factor in the oxidation processes of the egg. Any change brought about in these is immediately reflected in the oxygen uptake of the egg. Loeb has, of course, based his method of producing artificial parthenogenesis on the fact that alteration of the surface layer of the egg renders the commencement of development possible. But how can the cytolitic destruction of the surface layer of the egg lead to development? Warburg has shown that there are good reasons for believing that the oxidations taking place in the egg occur mainly at its surface, for NaOH, which does not diffuse into the egg, raises the rate of oxidations more than NH_4OH, which readily diffuses into the egg. Moreover, he found (11) that the addition of iron* salts to the broken up eggs, or acetone egg powder, was followed by a considerable increase in the oxygen consumption of these egg preparations. If the iron-content of the egg powder was doubled, the uptake of oxygen was also doubled. He found marked traces of iron in the sea-urchin egg. He suggests that the iron probably acts the part of a catalyser. If the iron were located in the lipoid layer of the egg in a condition in which it was unable to act, some slight alteration in this layer, due to the action of the sperm, might render it active or bring both the iron and the oxidisable substrate into a condition in which they could quickly interact. We know from Thunberg's work (12) that lecithin

* Warburg found 0·03 mgrm. iron per gramme dried egg substance.

in a watery suspension consumes considerable oxygen in the presence of iron salts. The egg of the sea-urchin contains considerable quantities of this lipoid.

Warburg (13) has pointed out that there are many points in which the metabolism of the fertilised egg resembles that of the yeast cell. In each it has been shown that structure plays a very important part, as acetone preparations of both the egg and the yeast-cell retain considerable respiratory power. Meyerhof (14) finds, however, that if acetone yeast is well washed with water, it soon loses its capacity to take up oxygen. If a little watery extract of yeast is added to the washed yeast, it immediately regains its lost respiratory power. In the water used in washing the yeast Meyerhof found the presence of some compound containing the (SH) group. Hopkins (15) has recently isolated from the yeast cell a substance which is undoubtedly closely related, if not identical with this respiratory body of Meyerhof. It proves to be a combination of two amino-acids, glutamic acid and cystine, to which Hopkins has given the name of glutathione. This dipeptide possesses most remarkable properties in that, in the reduced (SH) form, it can take up molecular oxygen, while in the oxidised (S–S) form so produced it can act as a hydrogen acceptor, and can catalyse oxidations of the Wieland type, in which no activation of oxygen probably takes place, but an activation of hydrogen occurs instead. In the presence of a suitable acceptor the hydrogen is removed and the oxidation of the original substance takes place. It can therefore be both reduced and oxidised under the influence of factors known to be present in the tissues themselves. Moreover, it possesses precisely those properties which a co-ferment adapted to an oxidase system would possess, and at present stands entirely in a class by itself. Hopkins has shown that it is present in most living cells, but he could find no trace of it in the hen's egg, although it was very obviously present in the 30-hour chick.

I find, however, that in the ripe eggs and sperm of *E. miliaris* it is invariably present in an appreciable quantity in the reduced form, but one minute after fertilisation the same eggs give a very deep magenta colour by the nitro-prusside test. It is very readily washed out of the eggs by warming them with a trace of acetic acid in sea-water: the washed eggs then no longer give the test. In the unripe egg, in which the nucleus is plainly visible, I could find no trace of its presence by the nitro-prusside test. In the ripe eggs it is present in the reduced form in very variable quantities, no two females giving the same result, probably depending on varying degrees of ripeness of their gonads. In a number of samples of ripe sperm it seemed to be present in much less quantity than in the eggs; but here again in two samples of sperm it was present in much greater quantity than in any of the eggs examined.

It is, of course, possible that the dipeptide is present in considerable quantity in the eggs and sperm in the oxidised (disulphide) form, and that during fertilisation it undergoes reduction. It has, of course, long been known that the hydrogen ion concentration of the sea-water exercises a marked influence on the uptake of oxygen by the egg of the sea-urchin. Thus Warburg (13) found that an increase in the HO concentration in the sea-water in which the eggs of *Strongylocentrotus* were placed from 10^{-8} to 10^{-3} increased the oxygen consumption of the eggs from 1·4 to 8·1. Hopkins finds that glutathione in the oxidised form is rapidly reduced by fresh tissues, but that this reduction is greatly accelerated if the reaction or P_H of the medium is well on the alkaline side of neutrality, while an acid reaction greatly retards this reduction. In a similar manner the oxygen uptake of the egg-cell is accelerated by alkali and retarded by acids.

That the glutathione is readily washed out of the eggs is shown by a slight pink colour the wash-water gives by the nitro-prusside test. In certain experiments in which the unfertilised eggs were treated so that their glutathione was washed out, I could find no trace of respiratory power on the part of these washed eggs. The same eggs unwashed showed well-marked respiration. The oxygen consumption of the unfertilised egg is so low, however, that it is perhaps unfair to assume on this ground that glutathione is the sole body concerned in the respiration of the ovum. Prof. Hopkins has been so kind as to undertake certain experiments with washed egg material for me, and he tells me that on the addition of glutathione to these egg preparations, the reduction of this in the presence of fresh tissue was markedly greater in the case of the fertilised washed eggs than in the case of the unfertilised. There seems to be fairly substantial ground then for believing that there is an immediate increase in the quantity of this remarkable body in the ovum on fertilisation.

It has been mentioned that cystin is one of the amino-acids entering into the composition of glutathione; it is interesting to note that Warburg (16) has recently drawn attention to the fact that cystin, absorbed on the surface of carbon particles, is capable of considerable respiration, taking up oxygen and giving off carbon dioxide. He found that one gram of blood carbon dissolved in a similar weight of a 1/500 N cystin solution, took up the same quantity of oxygen as a similar weight of liver tissue. The carbon-cystin system, moreover, under the action of oxygen, gives the same end-products as the combustion of egg-white, that is carbon dioxide, ammonia and sulphuric acid.

There are a number of other interesting points brought up by the presence of glutathione in the germ-cells of the sea-urchin and the possible *rôle* it may play in the oxidation processes of the ovum. There can hardly be much doubt, therefore, that its investigation in the future will reveal many new and hitherto unsuspected facts with regard to the oxidations taking place in the ovum on fertilisation.

V. SUMMARY.

1. By the use of a special type of the Barcroft differential manometer the oxygen consumption and the carbon dioxide output of the egg of *E. microtuberculatus* has been measured during the period the sperm are actually making their way into the egg. The eggs were fertilised in the closed chambers of the apparatus and their respiration observed before and during fertilisation.

2. It has been shown that during fertilisation the sperm within a minute of their being added to the eggs bring about an immediate increase in their oxygen consumption.

3. The study of sections of fixed material of the eggs during the process of fertilisation, shows that within 2 minutes of the sperm being added to the eggs they have not penetrated the egg membrane. They are only in contact with its external surface.

4. This contact of the spermatozoön with the external surface of the egg membrane, however, is capable of increasing in the space of a minute the oxidation rate of the ovum by something more than 8000 per cent.

5. During fertilisation there is more oxygen taken up in the first minute of the process than at any subsequent interval of the same time.

6. The carbon dioxide output of the eggs during fertilisation closely follows the oxygen consumption, the respiratory quotient varying from 0·9 to 0·95.

7. The curve of the oxygen consumption of the ovum during fertilisation points conclusively to the stage when the sperm are in contact with the egg membrane as the most important part of the process.

8. The fusion of the male and female pronuclei in the later phases of fertilisation is correlated with no additional increase in the oxygen consumption of the egg-cell.

9. The oxidation rate of the ovum on fertilisation is probably considerably greater than that of any of the adult body tissues, while the oxidation rate of the mature unfertilised ovum is very much less than that of any adult body tissue.

10. The ripe sperm and eggs of *E. miliaris* contain appreciable quantities of the dipeptide *glutathione* in the reduced (SH) form. In the egg 1 minute after fertilisation it is found in much greater quantity than in the unfertilised condition. It can be washed out of both the fertilised and unfertilised egg.

The washed eggs no longer give the nitro-prusside test. In the immature germ cells it seems absent, at least in the reduced form.

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The Depressor Nerve of the Rabbit.

By B. B. Sarkar.

(Communicated by Sir E. Sharpey Schafer, F.R.S. Received October 31, 1921.)

(From the Department of Physiology, Edinburgh University.)

[Plate 4.]

Since the discovery of the depressor nerve,* much work has been done in connexion with its important influence on the regulation of blood-pressure, but (so far as I am aware) no attempt has been made to determine its histological structure.

Origin and Course of the Depressor.

Cyon† gives the following description of the origin of the nerve. The depressor nerve in the animals worked upon usually begins with two branches at the point of departure of the superior laryngeal nerve from the vagus, one from each of the two nerves. The nerve soon after its origin passes towards the cervical sympathetic, in company with which it descends the neck towards the inferior cervical ganglion. With this ganglion it is often connected by fine branches : it then turns inward past the subclavian artery, and loses itself at the base of the heart, to which it passes from behind between the pulmonary artery and the aorta. Just before entering the heart tissue the two depressors lie close to one another.

This is stated by Cyon to represent the course of the nerve in rabbits, cats, and horses, and probably in other mammals in which the cervical sympathetic runs separately from the vagus.

My own observations have been made upon rabbits. I have not been able to substantiate the statement that a separate depressor is present in the cat, and I have had no opportunity of investigating the subject in horses.

Besides making a number of sporadic observations on animals killed for different purposes, I have examined the depressor systematically on both sides in seven rabbits. I find that it varies greatly in its mode of origin, which is usually, as stated by Ludwig and Cyon, from two branches, one from the vagus, the other from the superior laryngeal (fig. 1). But in some cases it was a single nerve throughout (fig. 2), while in others the two branches of origin ran separately for a greater or less distance, and even to their destination (fig. 3).‡ When single the nerve was generally found to originate from the angle where the superior laryngeal leaves the vagus. When the two branches of origin were separate, one usually left the vagus at this point, but sometimes lower down, the other was derived from the superior laryngeal a short distance after it had left the vagus. In certain cases the whole nerve ran along the superior laryngeal for a little distance before emerging as a separate nerve. As Ludwig and Cyon state, the depressor lies close to the cervical sympathetic throughout most of its course:

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* Ludwig and Cyon, Arbeit. physiol. Anstalt, Leipzig, 1866.

† 'Methodik d. physiol. Experimente,' 1876.

‡ Figs. 1, 2, and 3, are merely diagrams, and are not intended to represent the actual size, distance apart, or length of the nerves.

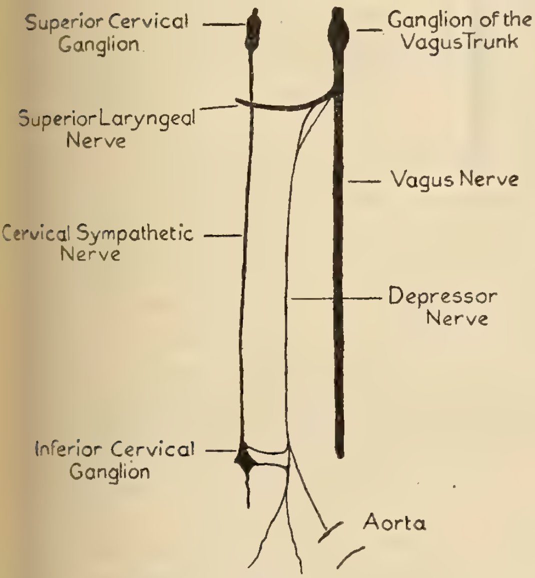
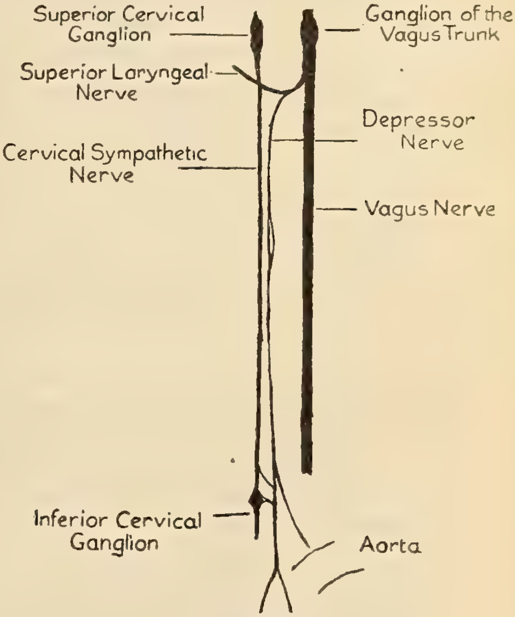

Superior Cervical Ganglion
Ganglion of the Vagus Trunk
Superior Laryngeal Nerve
Vagus Nerve
Cervical Sympathetic Nerve
Depressor Nerve
Inferior Cervical Ganglion
Aorta

FIG. 1.


Superior Cervical Ganglion
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Aorta

FIG. 2.

FIG. 1.—Diagram of origin and course of depressor nerve in a rabbit, showing the usual derivation of the nerve by two threads, one from the superior laryngeal and the other from the vagus trunk.

FIG. 2.—Diagram showing the nerve originating as a single filament from the superior laryngeal. Note the (unusual) splitting and rejoining of the nerve in the middle of its course.

in the lower part of the neck one or two short branches unite it with the inferior cervical ganglion. I have usually found it to divide at its lower end into three filaments, one of which passes to the aorta and the other two to the base of the heart. In two instances in which the two branches of origin were quite separate throughout, one branch (the vagal) ran to the aorta without forming any connexion with the inferior cervical ganglion,* while the other (the superior laryngeal) was connected by two filaments with that ganglion and then passed to the tissue at the base of the heart (fig. 3).

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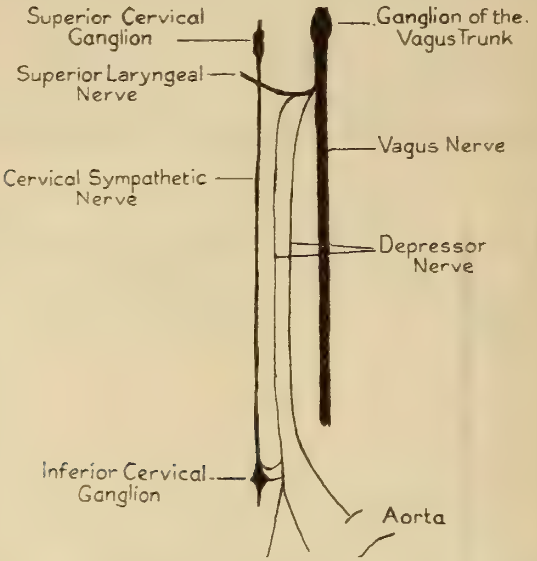
* This ganglion, which has always been known to physiologists as the "inferior cervical," is now frequently termed by anatomists the "middle cervical."


Superior Cervical Ganglion
Ganglion of the Vagus Trunk
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Cervical Sympathetic Nerve
Depressor Nerve
Inferior Cervical Ganglion
Aorta

Fig. 3.—Diagram showing the depressor passing down as two separate nerves, one of which, arising from the superior laryngeal, goes to the aorta; the other, arising from the vagus trunk, to the base of the heart. The first one is connected with the inferior cervical ganglion by two fine filaments. (These are also shown in figs. 1 and 2.)

To determine the exact origin of the nerve, serial sections of the vagus were made upwards from the angle where the superior laryngeal and the depressor leave it. All three nerves are encased in a single epineurium until the superior laryngeal separates from the vagus (fig. 6—see Plate 4). On examining such a series of sections a group of ganglion cells is found lying between the superior laryngeal and the main vagus trunk just before the superior laryngeal separates out (fig. 4). In some animals these cells are continued into the beginning of the superior laryngeal nerve as far as the point where its depressor branch leaves that nerve. When the whole depressor or one of its parts separates from the vagus after the superior laryngeal branch has been given off, the group of ganglion cells in question extends down the vagus trunk nearly as far as the point where its depressor branch emerges (fig. 5). These cells probably give origin to afferent fibres of the depressor. In cases in which the depressor emerges high up, the group may lie close below the ganglion trunci, although distinctly separate from that ganglion.

Microscopic Examination of the Nerve.

For histological purposes a piece of the nerve was slightly stretched on a card, fixed with osmic acid, washed and transferred to alcohol. Sections were cut by the paraffin method (figs. 7 and 8). Teased preparations were also examined. Microscopically, the nerve is found to consist of both myelinated and non-myelinated fibres (fig. 9). The myelinated fibres are both medium-sized and very fine. Most are between 4μ and 6μ (0·004–0·006 mm.) in diameter, but in two nerves two or three fibres of about 8μ (0·008 mm.) were found. The fine myelinated fibres have the character of pre-ganglionic autonomic nerves. The non-myelinated resemble post-ganglionic autonomic fibres. Both these kinds of fibres are presumably efferent.

The size of the whole nerve varies in different cases. The right nerve is usually smaller than the left and contains fewer myelinated fibres. I have made an attempt to count the myelinated fibres in six rabbits, but the small diameter of the finest fibres renders the task a difficult one. And it is impossible to see the non-myelinated fibres clearly enough in section to be able to count them. Nor is it easy to obtain sections so exactly transverse as to show each myelinated fibre distinctly. The following figures are therefore given for what they are worth:—

The total number of myelinated fibres of all sizes in the nerves of the two sides showed an individual difference of from 375 to 496 fibres, the average number being 433 in the two. The average number of myelinated fibres of all sizes in the right nerve was 177 and in the left 256. The total number of fibres contained in the branches when these remained separate fell within these limits.

Summary.

1. The depressor nerve of the rabbit appears to be connected, at least in part, with a special collection of ganglion-cells in the vagus, distinct from the ganglion of the trunk. This collection may extend a certain distance into the superior laryngeal, or may pass into the vagus trunk some distance below the ganglion of the trunk, but in most it lies in close contiguity with and just below that ganglion. The cells of the group in question probably give rise to the afferent fibres of the depressor.

The exact point of origin of the nerve is variable. It is usually formed by two branches, one from the superior laryngeal and one from the vagus. In some cases it is double throughout, in others single. It is connected below by fine branches with the inferior cervical ganglion, and can be traced to the root of the aorta and base of the heart.

2. The size of the nerve and the number of fibres it contains vary in different individuals. The left nerve is generally larger and contains more fibres than the right.

3. The depressor contains not only medium-sized myelinated fibres, but also a considerable number of very fine myelinated fibres, and others which are non-myelinated. It is, therefore, probably not wholly formed, as has usually been supposed, of afferent fibres, for these fine myelinated and non-myelinated fibres closely resemble those belonging to the autonomic nervous system and are presumably efferent.*

The expenses of this investigation have been mainly defrayed by a grant from the Earl of Moray Fund for promoting Research in the University of Edinburgh.

DESCRIPTION OF PLATE.

Fig. 4.—Section of vagus trunk just before the superior laryngeal branch is given off. On the left is seen the vagus; on the right, the superior laryngeal; between them the group of cells from which the afferent fibres of the depressor probably arise. Photograph × 75 diameters. Osmic preparation.

Fig. 5.—Section from the same preparation but taken a little lower down. The superior laryngeal is now quite separate from the vagus. The group of cells shown in fig. 4 is still visible, and fibres of the depressor are beginning to accumulate at the side of the vagus. Photograph × 75 diameters.

Fig. 6.—Section from the same preparation still lower down. The three nerves now appear as entirely distinct bundles, each surrounded with its own perineurium, but enclosed in a common epineurium. On the left, the vagus; on the right the superior laryngeal; the depressor is the small bundle of fibres between them. Photograph × 75 diameters.

Fig. 7.—Section of a depressor nerve. Osmic preparation. Photograph × 400 diameters.

Fig. 8.—Section of the two filaments of origin of a depressor nerve, just before their union. Photograph × 400 diameters. (This section is not from the same animal as that from which fig. 7 was obtained.)

Fig. 9.—From a teased osmic preparation of a depressor nerve. Photograph × 300 diameters. The figure shows medium-sized and fine myelinated fibres and a few non-myelinated fibres.

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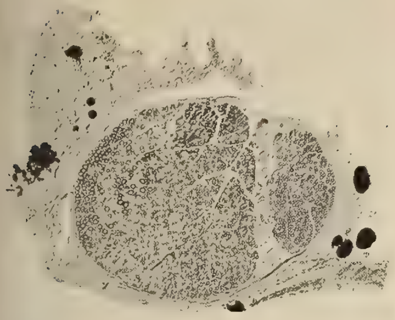
* According, however, to S. W. Ranson ('Physiological Review,' vol. 1, p. 479) small myelinated and unmyelinated fibres are included among visceral afferent fibres. This statement is based on his own observations recorded in 'Journ. Comp. Neurology,' vol. 29. See also the same Journal, vol. 24. (Private communication to E. S. S.)



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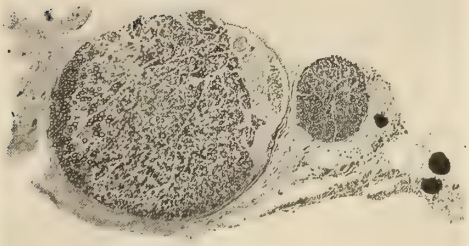

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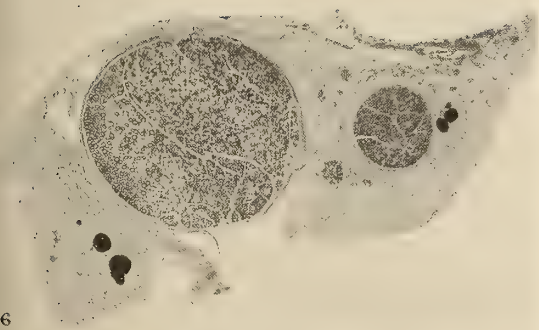

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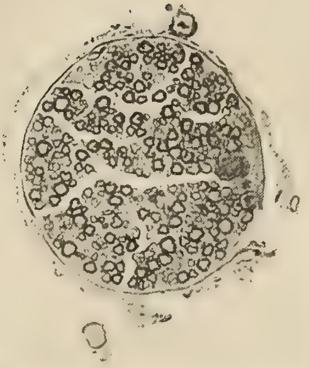

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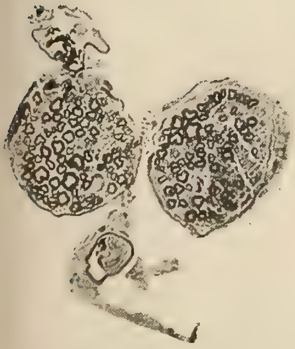

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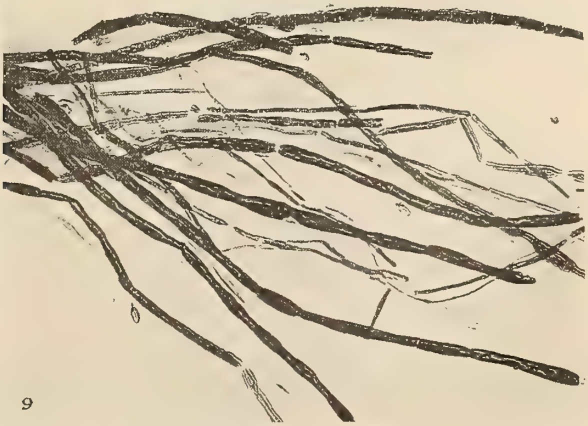

9

The Coagulation of Protein by Sunlight.

By Elrid Gordon Young, Ramsay Memorial Fellow.

(Communicated by Prof. F. G. Hopkins, F.R.S. Received July 6, 1921.)

(From the Biochemical Laboratory, Cambridge University.)

It was first shown by Dreyer and Hanssen (1) in 1917 that ultra-violet light produced a change in protein solutions which appeared to be similar to coagulation by heat. They exposed various solutions in quartz chambers to the light of a Bang lamp with iron and silver electrodes. Vitellin was found most easily coagulated, while globulin, albumin and fibrinogen showed a decreasing sensitivity to ultra-violet rays in the order mentioned. These investigators also discovered that acids markedly increase the rate of precipitation. Soret (2) had shown in 1883 that there are absorption bands in the extreme ultra-violet region of the spectrum of various proteins, *e.g.*, casein, ovalbumin, mucin and globulin. Tyrosine likewise has this band in the ultra-violet and Soret attributed to this constituent of the protein molecule its power of absorbing ultra-violet rays. In this connection Harris and Hoyt (3) carried out some interesting experiments on the protective power of various substances for paramœcium cultures exposed to ultra-violet radiations. They found that gelatin peptone, amino-benzoic acid, cystine, leucine and especially tyrosine possessed the power of detoxicating ultra-violet rays when placed as a thin layer of aqueous solution over paramœcium cultures under a quartz-mercury lamp. The toxicity of the radiations for paramœcia or protoplasm in general can be understood in the light of the discovery of Dreyer and Hanssen coupled with that of Soret.

From a physico-chemical standpoint Bovie (4) has published a study of the coagulation of proteins by ultra-violet light. By exposing solutions of crystalline ovalbumin, both dialysed and containing electrolytes, to the light of a mercury-vapour lamp, he came to the conclusion that there were two reactions involved in the coagulation of ovalbumin by ultra-violet light. The first is a photochemical one with a low temperature coefficient,—denaturation; and the second is one with a higher temperature coefficient of two and is dependent upon the electrolytes present,—coagulation. While using solutions dialysed against tap water Bovie made the observation that the protein appeared to become sensitive to light of longer wave-length, for his control tubes in glass were slowly coagulated.

Finally, from a medical point of view Schanz (5) has carried out a few experiments on this phenomenon with egg-white, blood serum and pig lenses.

From qualitative experiments only he claims to have discovered that certain substances, such as glucose, alcohol, lactic acid, urea and acetone, sensitise the serum proteins, while he states that certain German mineral waters protect them. He uses these observations to explain certain pathological conditions, especially old-age cataract.

When working with solutions of serum albumin which had been several times recrystallised, I had noticed that a marked change was produced in the solution while in glass vessels, so that the protein was almost quantitatively precipitated by mere exposure to sunlight. This precipitation occurred both with material which had been prepared by the alcohol-ether method and that obtained by direct crystallisation from serum, as I have described in a previous communication (6). A heavy precipitate appeared inside of 2 hours which proved soluble only in dilute alkali. The phenomenon seemed of such importance that a more thorough examination of the nature of the chemical reaction underlying it was undertaken.

As I have been unable to find any record of such a marked change in protein solutions by the action of sunlight in the literature, and further as the reaction was obtained with serum albumin of a purity such as has never, I venture to think, been achieved before, I attributed my observation of light instability to the purity of the preparations.

The following pages of experimental work are designed to show that the coagulant action of ultra-violet light on albumin in aqueous solution is also brought about by the visible rays of the spectrum when highly purified material is used. Experiments are described which show further that the nature of the reaction is identical with coagulation by heat in all points tested experimentally. That there are two reactions involved is demonstrated and changes in some physical constants of the pure protein solutions by the action of visible light rays are described as evidence towards an explanation of the primary reaction of protein coagulation.

METHODS.

Dialysis.—For the complete and rapid removal of contaminating electrolytes from solutions of albumin, the dialysing apparatus devised by Sörensen (7) has been used in a slightly modified form. It was necessary that the apparatus should possess the particular advantage of allowing the operator to maintain the solutions at their original concentration or to further concentrate them during dialysis.

The Preparation of Collodion Membranes.—Medicinal cotton wool was purified by extraction with boiling dilute alkali, followed by repeated changes of boiling water. The cotton was then dried and further extracted in refluxing absolute alcohol. After drying nitration was carried out as recommended by Blitz and von Vegesack (8). The washed and dried cellulose nitrate was dissolved in anhydrous alcohol-ether mixture (3 : 1) to form a 3 per cent. solution. This was applied by the Sörensen technique to a test-tube of $\frac{3}{4}$ inch diameter. Six or seven applications were given with a drying interval of 10 minutes between applications, and a final drying period of 5 to 7 hours before soaking in water.

The Dialysing Apparatus.—The general arrangement of the various parts was essentially that of Sörensen, although a smaller, slightly modified cell was used. The latter had a capacity of 250 c.c., while the membrane sack held 40 c.c. By the insertion of an extra limb in the upper part of the cell, very close to the neck, it was possible to remove the dialysate completely from the lower opening by the admission of air through the extra limb before introducing fresh water. This, I have found, allows of more rapid removal of the electrolytes present with a minimal use of distilled water, and also of the ready withdrawal of samples of the dialysate for analysis. The dialysis was carried out at room temperature, *i.e.*, 10–15° C., and the negative pressure found necessary varied from 5 to 20 cm. of mercury. The dialysate was replaced with distilled water twice daily. The only satisfactory way of determining whether the dialysing apparatus was in proper working order was to carry out a series of analyses on the successive dialysates withdrawn. Table I shows how efficiently the cell was functioning when it contained a solution of ovalbumin of 11·09 per cent. strength. After the fifth day no further positive test for the predominant impurity, $(NH_4)_2SO_4$, could be obtained. The solution contained 1·440 grm. $(NH_4)_2SO_4$ at the start and the first two days' dialysis served to remove more than 98 per cent. of the contaminating sulphate.

Table I.—Dialysis of Ovalbumin Solution, 11·09 per cent.

Day.	SO_4 test.	Protein test.	$(NH_4)_2SO_4$ content.	
			Grm.	Per cent. total.
1	+ + + +	—	1·2675	88·02
2	+ +	—	0·1473	10·23
3	+	—	0·0187	1·30
4	+	—	0·0023	0·16
5	—	—	0·0007	0·05
			1·4365	99·76

The $(NH_4)_2SO_4$ was determined by distillation with aeration as in a micro-Kjeldahl determination, using N/20 or N/100 H_2SO_4 in the distillate receiver and titrating excess acid by N/20 or N/100 $Na_2S_2O_3$ in the iodide-iodate titration.

The Preparation of Albumin.—Crystalline ovalbumin from one dozen egg whites was prepared by means of $(NH_4)_2SO_4$ and N/3 H_2SO_4, crystallising and recrystallising at the isoelectric point. Samples of the successive recrystallisations were dialysed in the apparatus previously described and the remainder after the third crystallisation treated likewise.

About 800 c.c. of horse serum were treated as described in a previous communication (6), using the direct method, and the crystals obtained recrystallised three times. This material was used for several of the experiments described below.

Technique of Light Exposure.—Small test tubes of $\frac{5}{8}$ inch diameter of ordinary clear glass were used. About 5 c.c. of the fluid under examination were placed in a test tube or a small glass spectroscope box and exposed to sunlight or artificial light from which the infra-red rays had been removed by passage through a vessel with parallel sides containing clear water. A 5-inch lens with a focal length of 6 inches was used to concentrate the light rays, and it was placed about 5 inches distant from the light source in the case of the arc. The entire solution was exposed to the rays somewhat in advance of the focal point.

It was highly desirable to obtain a sufficiently powerful source of artificial light with some degree of constancy in place of the capricious sun. The light from a carbon arc, made by the Firma Carl Zeiss for use with their ultra-microscope and mounted on an optical bench, was used for several experiments. A current of 10–15 ampères was employed. This will bring about the same effect as sunlight if exposure be for a sufficient length of time. The intensity of the arc is only one-tenth that of mid-day sunlight, and thus an approximately similar result by artificial light requires about five times or more the length of exposure to sunlight. Another disadvantage in the use of the arc is the necessity of changing carbons frequently, for with the current strength used the carbons are completely burned up in $1\frac{1}{2}$ hours, thus necessitating changing three or four times in the course of an experiment with ovalbumin.

Method of P_H *Determinations.*—The concentration of hydrogen ions in the various solutions used was determined colorimetrically, using the standard buffer mixtures devised by Clark and Lubs (9). The accuracy of the buffer mixtures was checked electrometrically by means of the potentiometer and the Barendrecht electrode. After some experimentation, the three following indicators were selected as most suitable for the purpose.

P_H.	Indicator.
3·1—4·4	Methyl orange.
4·4—6·0	Methyl red.
6·4—7·8	Neutral red.

To obviate possible errors due to the presence of proteins and salts, the method of dilution was adopted. Thus 1 c.c. of fluid was pipetted into a test tube, 4 c.c. of CO_2-free water added, and a suitable quantity of the indicator. A comparator was employed in cases of turbidity or foreign coloration. The procedure of dilution was tested and the values obtained by the use of the indicators were verified electrometrically on test solutions.

RESULTS.

Experiment 1.—*Susceptibility of different Crystallisations to Light Change.*

A series of test tubes were prepared containing solutions of the first three crystallisations of ovalbumin. These were approximately of 2 per cent. strength and had been dialysed free from electrolytes. They were adjusted to the same P_H by addition of a few drops of N/10 HCl. Another series was prepared containing 1 c.c. of the different ovalbumin solutions and 2 c.c. of a buffer mixture, made up of equal volumes of N/1 CH_3COOH and N/1 CH_3COONa, giving a P_H of 4·74. The results are shown in Table II.

Table II.—Susceptibility of Different Crystallisations to Illumination.

Test.	Mixture.	P_H.	Result.
1	3 c.c. solution, 1st crystallisation	5·4	–
	1 c.c. „ „ + 2 c.c. buffer...	4·6	–
2	3 c.c. „ 2nd „	5·4	–
	1 c.c. „ „ + 2 c.c. buffer...	4·7	+
3	3 c.c. „ 3rd „	5·4	–
	1 c.c. „ „ + 2 c.c. buffer...	4·7	+ +

Time of exposure to sunlight, 6 hours.
Albumin concentration of unbuffered solutions, 2 per cent.

From the results recorded in Table II, where precipitates appeared in the buffered solutions of the second and third crystallisations of ovalbumin, it became evident that repeated crystallisation tended to render the albumin more sensitive to light. It is to be noted that no precipitate appeared in the unbuffered solutions. Now, between the unbuffered and buffered solutions there existed three differences. The buffered solutions possessed a slightly lower concentration of protein, a slightly higher concentration of hydrogen ions, and a much greater electrolyte-content than the unbuffered solutions.

The precipitate obtained was soluble in alkali but insoluble in excess water or acid. The amount was small, and only a low fraction of the total protein in solution.

Experiment 2.—*The Influence of* P_H *Variation on the Light Reaction.*

To determine the possible effect of P_H variations, a series of tests were prepared containing ovalbumin solution of the third crystallisation product, to which a few drops of acetic acid of 10 per cent. strength had been added. A control tube containing the same albumin solution, with buffer mixture, was illuminated at the same time. Further controls of tests 1 and 5 were prepared, and kept at the same temperature in the dark. The proportions and results are recorded in Table III.

Table III.—Effect of P_H Variations on Unbuffered Ovalbumin Solutions.

Test.	Mixture.	P_H.	Result.
1	5 c.c. solution + 2 c.c. buffer solution	4·8	+
2	5 c.c. „ + 0·05 c.c. acetic acid	4·7	−
3	5 c.c. „ + 0·10 c.c. „	4·6	−
4	5 c.c. „ + 0·15 c.c. „	4·45	−
5	5 c.c. „ + 0·20 c.c. „	4·2	−

Time of exposure to sunlight, 8 hours.
Concentration of ovalbumin, approximately 2 per cent.

The P_H values were determined at the close of the experiment, and the results were only seen in reality when the tubes were all brought to the isoelectric point of ovalbumin. On neutralising the acidic solutions with dilute NaOH, a marked precipitate appeared, about P_H 5·0 (4·8–5·4), which was soluble in excess of alkali, but reprecipitated at the same P_H on adding acid. This precipitate remained undissolved on further acidification of the solution. The volume of the precipitate varied directly with the original degree of acidity. The two unilluminated control tubes showed no precipitate whatever when adjusted to a P_H of 5·0. This experiment shows that acid increases the rate of the light reaction, but that in solutions very low in their concentration of electrolytes, the albumin is not precipitated. This observation brings the reaction in very close similarity to heat coagulation. The absence of a precipitate in the unbuffered solutions of Experiment 1 can now be explained, in the light of the fact brought out by Experiment 2.

A wider range of P_H variations was next tried, with the purpose of discovering the influence of alkali on the reaction. Phosphate and phthalate buffer mixtures were employed, with a solution of ovalbumin three times recrystallised. The time of exposure to very bright sunlight was 5 hours. P_H determinations were made both before and after the exposure. The results are recorded in Table IV. Each test consisted of 2 c.c. of buffer and 1 c.c. of ovalbumin solution. The concentration of ovalbumin in the original solution was 2 per cent., and in the illuminated tubes was thus about 0·7 per cent. Controls of tests 1 and 6 were prepared and kept in the dark, with negative results.

Table IV.—Effect of P_H Variations on Buffered Ovalbumin Solutions.

Test.	P_H.		Result after exposure.	At P_H 4·8.
	Before.	After.		
1	3·0	3·5	+ + + +	+ + + +
2	4·0	4·3	+ +	+ +
3	4·8	5·1	+	+
4	5·4	5·6	opalescent	+
5	6·0	5·6	”	+ +
6	7·6	6·5	”	+ + + +

Time of exposure to sunlight, 5 hours.
Concentration of ovalbumin, 0·7 per cent.

At the conclusion of the period of illumination, the tubes on the acid side of P_H 5·4 showed precipitates, the degree of which varied directly with the hydrion concentration. The tubes on the alkaline side of P_H 5·4 were merely opalescent. On acidulation to a P_H of 4·8, however, there appeared precipitates, the degree of which varied directly with the hydroxyl ion concentration of the original solution. The comparison between heat coagulation and light coagulation is thus brought even closer. Both acids and alkalies increase the rate of the light reaction, and during this change there is a removal of H or OH ions from the solution depending upon its reaction.

Experiment 3.—*What Physical Changes does the Protein undergo before Flocculation?*

In order to discover whether some physical change in the solution which was being subjected to light bombardment might be used as an indication of the rate of the light reaction, several properties were investigated. It was my intention to study the first reaction quite separately, if possible, from the second, involving the flocculation of the altered protein.

(A) *Optical Rotation.*—A 100-mm. tube, filled with the dialysed solution of the third crystalline product of ovalbumin, was exposed to the light of the arc, and readings of the optical rotation were made frequently by removing the tube to a Hilger polarimeter, with direct-vision spectroscope, and observing the rotation (α_E) of the green line of a quartz mercury-vapour lamp ($\lambda = 546{\cdot}1\ \mu\mu$). The instrument was accurate to 0·01°. The observations were as follows:—

Table V.—Effect of Illumination on Optical Rotation.

Time of illumination.	α_E.	$[\alpha]_E$.
minutes.	°	°
0	0·79	−36·60
10	0·81	−37·60
30	0·84	−38·99
45	0·87	−40·38
105	0·87	−40·38

Concentration of ovalbumin, 2·16 per cent.
α_E = observed rotation.
$[\alpha]_E$ = specific rotation.

The results in Table V show that one of the effects of radiations of the visible spectrum on pure crystalline ovalbumin is an increased power of optical rotation. This increase in specific rotation was observed in a solution that was entirely free from precipitate. A control tube of the same length, and containing the same solution, was read consecutively with the illuminated tube. It gave the same reading of 0·79 throughout the experiment.

An experiment to confirm the above observation was tried, using crystalline serum albumin which had previously been exposed to sunlight for 2 hours. The protein thus exposed was completely coagulated, for the solution contained $(NH_4)_2SO_4$ to the extent of 1 per cent. It is to be noted that in this experiment the serum albumin of 3·46 per cent. strength was completely coagulated inside of 2 hours, whereas ovalbumin in similar concentration is only partially coagulated after 6 to 8 hours' exposure. Now, the original unilluminated serum albumin had had a specific rotation of −78·60° by the green line of the mercury spectrum ($\lambda = 546{\cdot}1\ \mu\mu$). To the coagulum, with its supernatant liquid of about 100 c.c., were added fifteen drops of concentrated ammonia, and complete solution was thus brought about. This solution was examined in the polarimeter, and the specific rotation of the serum albumin was found to have risen to almost 5° above that of the undenatured substance. Now, in the case of unilluminated ovalbumin, I have been able to show that the specific rotation is a constant only at the isoelectric point (6). If acid be added, the value for [α] is increased. If alkali be added, the value is temporarily decreased. In order to discover whether this was also a fact with the denatured albumin, two or three drops of HCl (20 per cent.) were added, so as to adjust the P_H of the solution near to the isoelectric point. The specific rotation rose almost 5° to a constant value of −87·92° in several hours. The solution was again made alkaline by means of a few drops of ammonia and again the value for the specific rotation fell to −83·0°. The same slow rise to the higher value was observed as in the case of unilluminated ovalbumin. It is to be noted that the fluctuations from −83·0° to −89·6° are around a much higher mean value than the one for undenatured material, −78·6°. The significance of this observation is discussed in a later section. Table VI gives the data in detail.

Table VI.—Optical Rotation of Denatured Serum Albumin.

Time interval.	Solution.	Temperature.	P_H.	α_E.	$[\alpha]_E$.
		°		°	°
—	Original	15	5·6	−2·71	−78·6
—	Denatured + NH_3	14·2	7·4	−2·88	−83·3
1 hour	„ + HCl	15	5·4	−3·01	−87·1
5 hours	„	15	5·4	−3·04	−87·9
12 hours	„	14·5	5·4	−3·04	−87·9
5 mins.	„ + NH_3	15	7·3	−2·87	−83·0
2 days	„	15	7·3	−2·95	−85·3
3 days	„	14·5	7·3	−3·09	−89·4
1 day	„ + NH_3	14·5	7·6	−3·10	−89·6
1 day	„	14·2	7·6	−3·09	−89·4

Concentration of serum albumin, 3·46 per cent.
Time of previous exposure to sunlight, 2 hours.

(B) *Viscosity and Surface Tension.*—A solution of ovalbumin, three times recrystallised and dialysed free from sulphate, was used in the following experiments. Changes in viscosity were measured by an Ostwald viscosimeter immersed in a thermostat kept at 20° C. and regulated so that the temperature was constant to 0·1°; 5 c.c. were used for a determination, measured by means of a calibrated pipette.

The surface tension was measured by means of a Traube stalagmometer (Gerhardt No. III), standardised by distilled water and found to give 37·10 drops. The instrument was accurate to 0·05 of a drop. It should be pointed out here that in such a measurement the tension measured is between air and the dispersion medium, not between dispersion medium and disperse phase. Any change the latter might undergo, however, would very probably be shown in the former, though not of necessity.

The results obtained as shown in Table VII indicate very small changes. It is well to point out that the amount of albumin undergoing change is very small relative to the amount present. This I have repeatedly shown by heat coagulation of the remaining solution at its isoelectric point. The arc light was used in this experiment and the solutions exposed in glass spectroscope boxes.

Table VII.—Effect of Illumination on Viscosity and Surface Tension.

Exposure time.	Viscosity.	$\eta \times 10^3$.	Surface tension.	γ.
hours.	seconds.		drops.	
0	19·80	11·75	39·00	69·74
1·5	20·60	12·23	39·10	69·56
3·0	20·80	12·34	39·60	68·70
4·5	21·00	12·47	39·70	68·52

Concentration of ovalbumin, 2·10 per cent.

There is thus a slight decrease in surface tension (γ) and a slight increase in viscosity (η), but the changes are so slight that as a means of following the rate of reaction they are unsuitable. It is, however, significant that such changes occur, indicating as they do underlying chemical changes while the denatured particles remain dispersed. The work is being repeated with serum albumin solutions.

Experiment 4.—*The Effect of various Substances on the Rate of Denaturation.*

For the following tests a solution of serum albumin, twice recrystallised, was used. It still contained the mother liquor adherent to the crystals, so that there was an appreciable quantity of $(NH_4)_2SO_4$ present (about 1 per cent.). This solution was quickly affected by sunlight, depositing a massive precipitate in an hour if the sunlight were intense. It was denatured by the arc light on 2 hours' exposure. The precipitate was found to be soluble in excess water, acid or alkali. The solution in water or acid could only be brought about with precipitates formed quickly and freshly deposited. This observation is comparable with that of Michaelis and Rona (10) on heat coagulation of serum albumin. If the precipitate forms slowly, its solubility in both acid and water is lost. I am not able to say whether the resolution is an indication of a truly reversible reaction, or whether it merely indicates on the part of the colloid the ability to reassume a charge and disperse itself, as is the case of pure gelatin, about its isoelectric point. Michaelis and Rona state that in the case of heat coagulation it is a true reversion, but adduce no evidence for their belief. In this connection it is interesting to recall the observation of Corin and Ansiaux (11) that if freshly coagulated egg albumin be vigorously shaken it is again dispersed and a clear solution results. This point is discussed under the theory of the whole phenomenon.

The effect of adding various substances was tried on the serum albumin solution, and control tubes were prepared in each case and kept in the dark at the same temperature. Electrolytes in moderate concentration were found to increase the rate of denaturation, *e.g.*, NaCl, $(NH_4)_2SO_4$, KSCN. Traces of alcohol, acetone and toluene did likewise. Glucose and ether had no apparent effect. To be in a position to study the effect of various substances, however, on the rate of coagulation, a quantitative method would have to be devised. From the above experiments it would appear as if any substances with dehydrating power would increase the rate of denaturation. I am at present working upon this subject.

The Nature of the Light Reaction.

From the foregoing experiments it will be apparent that the reaction brought about by sunlight is very similar to heat coagulation, if not identical with it. The main facts associated with the phenomenon of heat coagulation may be briefly summarised for purposes of comparison. Chick and Martin (12) have conclusively demonstrated that, under certain circumstances, *i.e.*, when the water present is in large excess and the hydrogen ion concentration is kept constant, the reaction can be proved to be of the monomolecular order. The reaction is between protein and water and the effect of temperature is merely to accelerate it. It has an extraordinarily high temperature coefficient and the velocity is influenced by a number of conditions, especially acid and alkali. During coagulation, if the reaction be on the acid side of the iso-electric point, hydrogen ions are removed from solution; if the reaction be on the alkaline side hydroxyl ions are removed. The degree of removal of the H or OH ions is dependent on the total concentration of acid or alkali and the P_H of the solution. Heat coagulation of albumin consists of two processes: (1) a reaction between protein and water (denaturation); (2) the separation of the altered protein in a particulate form (agglutination). From the investigation of Sörensen and Jürgensen (13) on ovalbumin, and of Michaelis and co-workers (14) on serum albumin, the maximum flocculation of denatured protein has been demonstrated to occur only at its isoelectric point. It is interesting to note here that Michaelis and Davidsohn (15) have found that the isoelectric point of denatured serum albumin (P_H 5·4) is not the same as natural serum albumin (P_H 4·6). Hardy (16) recognised the double nature of heat coagulation of egg white as long ago as 1899 and considered the primary change as one of an emulsoid colloid to a suspensoid type, which was readily precipitated by small quantities of electrolytes. Michaelis endorsed this view from his work on serum albumin. Most workers have confined themselves to a study of the conditions governing flocculation, and the more fundamental primary change from a natural protein to one which is readily precipitated on neutralisation of the charge carried by the particles has been neglected.

The explanation of coagulation generally accepted is due to Hofmeister and Pauli. They conceive of the primary change as one of dehydration involving the internal neutralisation through the loss of the elements of water of terminal NH_2 and COOH groupings. From a study of the coagulation of casein by alcohol Robertson comes to the same conclusion.

Now in the change brought about by sunlight there are two reactions. The first change involves a simple chemical one, for which the light is responsible, which causes an increase in optical rotatory power and in viscosity, a decrease in surface tension and a decrease in H or OH ions depending on the reaction of the medium. The second stage will only precede if the solution is at or near the isoelectric point. It is materially aided by small amounts of electrolytes or dehydrating agents such as alcohol or acetone. Under certain circumstances the second stage is reversible. The explanation would seem to lie in the ability of the albumin to reassume charged ions so long as agglutination has not proceeded too far. In other words the forces of repulsion of similarly charged ions attached to the colloidal particles are sufficient to overcome the adhesive forces of the albumin flocculi. It is conceivable that in the primary stage not only do we have internal anhydride formation within single colloidal aggregates, but that as coagulation proceeds this anhydride formation extends to linkages between two or more colloidal aggregates. The determination of H or OH ions reveals a diminution during coagulation. This can be readily explained on the basis of the amphoteric nature of the protein. On the acid side of the isoelectric point the protein is an acid and must possess some free COOH groupings. If these become neutralised, as in anhydride formation, the acidity of the solution will be diminished. The converse will hold true in alkaline solution where NH_2 groups function as basic influences.

The explanation of the primary reaction as simply involving an internal dehydration seems most reasonable on account of the great number of ways in which denaturation can be brought about. Light, heat, mechanical shock, undue strain as in the surface of expanding air bubbles, or any marked double phase such as is produced by the mixing of two immiscible liquids, acidity, alcohol, all these agents bring about a change such that the protein becomes insoluble. The action of alcohol is interesting in that in this reagent we possess one which can bring about both changes, but that if conditions are regulated only one will take place. Thus it is possible to remove the water of solution or solvate water from the colloidal particles by means of 95 per cent. alcohol in the cold. Flocculation is thus brought about, as is the case by concentrated salt solutions, which is reversible and not accompanied by the change called denaturation. If, however, alcohol be added to a protein solution in like manner, but at 30° C., then the power of alcohol as a dehydrating agent appears so augmented that not only does it remove solvate water, but also may be pictured as inducing internal anhydride formation (denaturation). The precipitate thrown down is irreversible. In the case of mechanical coagulation, the phenomenon is much more readily produced if a dehydrating agent is present in quantity. It would be interesting to know if it would occur at all should every trace of electrolyte be removed from the solution.

From the above discussion, it would appear that the *rôle* of light as a coagulating or denaturating agent is similar to that of heat—a catalyst of the primary fundamental chemical reaction. That certain substances can aid or hinder its action is very probable from preliminary observations. Certain it is, from the results of Experiments 3 and 4, that serum albumin is many times more sensitive to light than ovalbumin. In this connection, it is interesting to note that the purest serum albumin solutions still contained a minute amount of pigment. I am unable to say whether the greater sensitivity of serum albumin was or was not due to its influence.

SUMMARY.

Serum albumin and ovalbumin which have been several times recrystallised become sensitive to intense light, either sunlight or strong arc illumination, from which the infra-red and ultra-violet rays have been removed. Serum albumin is many times more easily affected than ovalbumin.

The change brought about by light has many of the characteristics of heat coagulation. It consists of two separate reactions: (1) denaturation—a primary chemical change; (2) flocculation—the precipitation of denatured particles.

The primary reaction is accompanied by increase of viscosity and optical rotatory power, and decrease of surface tension. The velocity of the primary change is increased both by acids and by alkalies. During the reaction, H ions are removed if the P_H be on the acid side of the isoelectric point; if on the alkaline side, OH ions are removed.

The secondary reaction does not follow if the solution be free from electrolytes. It is brought about by adjusting the solution to about the isoelectric point of the albumin, P_H 4·8–5·4. Under conditions when the precipitate is formed readily, it will go back into solution on the addition of acid or excess water. If the precipitate is formed slowly, it is only dispersed by alkali.

Certain substances act as accelerators for the reaction, such as alcohol, acetone, $(NH_4)_2SO_4$, NaCl, KSCN. The mechanism of the reaction is discussed.

In conclusion, I wish to take this opportunity of expressing my thanks to Prof. F. G. Hopkins for much kind criticism and encouragement.

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On the Development and Morphology of the Leaves of Palms.

By Agnes Arber, D.Sc., F.L.S., Keddey Fletcher-Warr Student of the University of London.

(Communicated by Prof. F. W. Oliver, F.R.S. Received October 8, 1921.)

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I. Introduction.

In series of previous memoirs* I have discussed the phyllode theory of the Monocotyledonous leaf, both in general and in relation to a number of special cases. In the present paper I propose to consider the Palms, with a view to determining whether their highly peculiar leaf structure is open to interpretation on the lines which have suggested themselves in the course of my study of other families, in which the leaves are less obviously anomalous.

The mature Palm leaf consists of a closed basal sheath (sometimes continued upwards into an ochrea), a leaf-stalk, and a limb, which may be of palmate or pinnate form; the "fan" and "feather" types of limb grade into one another, the distinction depending only on the degree of elongation of the median rachis. The Fan-palms differ from the Feather-palms in one further point on which great stress is sometimes laid—namely, that they bear small excrescences at the base of the limb. The outgrowth on the ventral side is the so-called ligule; less frequently a corresponding but smaller structure, known as the dorsal scale, occurs on the opposite side.

The morphological value of the various members which constitute the Palm leaf will be discussed in a later section of this paper (pp. 257-60).

It has long been known that the "compound" appearance of Palm leaves is the result of secondary changes, and that these leaves are thus not truly equivalent to the divided or compound leaves of Dicotyledons. Nearly

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* Arber, A. (1921), and earlier papers there cited.

a century ago, A. P. de Candolle* pointed out that the young leaves of Palms are entire, and that it is only subsequently that they become torn into thongs (" déchirées en lanières "); the segments are thus merely pseudo-lobes (" prétendus lobes "). De Candolle emphasises the fact that, though the idea of cutting (" découpure ") enters so largely into the technical language of leaf-description, it is only to the Palms that such terms can be applied with literal exactitude.

Subsequent workers have carried back our knowledge of Palm leaves to the earliest stages in their developmental history. Von Mohl,† Karsten,‡ and Trécul,§ in the middle years of the nineteenth century, followed the course of their entogeny, and attempted to trace the stages passed through by the leaf-rudiment between its origin and that curiously plicate phase which is succeeded by the ultimate subdivision into separate segments. About forty years later, the subject received renewed attention, principally from Eichler‖ and Naumann;¶ the work of Eichler remains, even to the present day, the standard account of the development of Palm leaves. More recently, Deinega** and Hirmer†† have returned to certain aspects of the question which the earlier workers had left in some obscurity.

A study of the literature shows that, though there is essential unanimity as to the manner in which the simple leaf-limb splits into its ultimate pseudo-compound form, there are, on the other hand, marked differences of opinion about the history of *that early developmental period which precedes the actual subdivision of the leaf.* All workers are agreed that, at an extremely early stage, the upper part of the leaf-rudiment shows a series of deep furrows alternating with ridges, which give it an apparently plicate form. But authorities differ as to the mechanism by which this plication is brought about. One school has maintained that there is actual *splitting* of the leaf-tissues (von Mohl, Trécul, Naumann), while others hold that only *folding* takes place (Karsten, Eichler, Deinega, Hirmer). The discussion of these conflicting views will be postponed until after I have described my own observations on the ontogeny of the Palm leaf.

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* Candolle, A. P. de (1827).

† Mohl, H. von (1845).

‡ Karsten, H. (1847).

§ Trécul, A. (1853).

‖ Eichler, A. W. (1885).

¶ Naumann, A. (1887).

** Deinega, V. (1898).

†† Hirmer, M. (1919); see this paper for further references.

II. THE ONTOGENY OF THE PALM LEAF.

1. *The "Plicated" Limb.*

In my study of the Palms I have not confined myself to genera whose leaf development has hitherto remained undescribed, but I have examined every example of which I could obtain material, because I found that, as my standpoint differed somewhat from that of previous workers, the existing descriptions did not settle the questions with which I was particularly concerned. I have followed the mode of development of the young leaves in representatives of four of the five sub-families of the Palms, but in the case of the fifth group—the anomalous Phytelephanteæ (*Nipa* and *Phytelephas*)—I regret that I have been unable to obtain material. Most of my observations relate to the ontogeny of the leaves of seedlings of various ages, because it is almost impossible, in this country, to procure many apical buds of fully developed Palms. The plumular leaves are generally of simpler form than the leaves of the mature plant, but there is no reason to suppose that they differ from them in any essential respect in the early stages of ontogeny.

The species which I have examined are distributed as follows among the different tribes:—

I. CORYPHINEÆ.	
Phœniceæ	*Phœnix canariensis*, Hort., *P. dactylifera*, L., *P. Rivieri*, Hort.
Sabaleæ	*Chamærops humilis*, L. *Pritchardia filifera*, Lind. *Rhapis humilis*, Blume. *Thrinax excelsa*, Lodd. *Trachycarpus* (*Chamærops*) *excelsus*, H. Wendl., T. (*C.*) *Fortunei*, H. Wendl.
II. BORASSINEÆ.	
Borasseæ	*Latania Commersonii*, J. F. Gmel.
III. LEPIDOCARYINEÆ.	
Calameæ	*Dæmonorops* (*Calamus*) *melanochætes*, Blume.
IV. CEROXYLINEÆ.	
Arecineæ	*Areca sapida*, Soland. *Bentinckia nicobarica*, Becc. *Howea Belmoreana*, Becc. *Oreodoxa regia*, H. B. et K.
Cocoineæ	*Cocos Romanzoffiana*, Cham.

I find that in all these species—Fan- and Feather-palms alike—the mechanism of development of the plicated or lamellated limb follows essentially the same lines. For the sake of brevity, I will limit myself to three cases as examples. In the Fan-palm, *Trachycarpus Fortunei* (fig. 1, A–F), the second plumular leaf, examined at a very young stage, was found to have at the base a closed sheathing region (fig. 1, A); this was succeeded by the petiole (fig. 1, B), an organ of roughly triangular outline with a concave upper surface. The petiole retained this characteristic form for a very short distance only (about 20μ), and then the lamellate structure was rapidly reached by the development of a series of grooves penetrating deeply into the leaf-stalk tissue between the vascular bundles (fig. 1, C–F). In fig. 1, E, I have numbered the grooves to show the order of their origin. The process of grooving is a very rapid one, the stages between fig. 1, B, and fig. 1, E, taking place within a distance of about 0·5 mm.

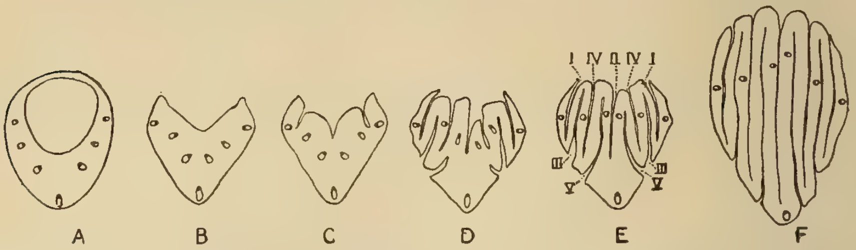

Fig. 1.—*Trachycarpus Fortunei*, H. Wendl. Series of transverse sections from below upwards through the second plumular leaf of seedling (×23). (Sections cut by Miss Ethel Sargant.) Fig. 1, A, sheath; fig. 1, B, petiole; fig. 1, C–E, process of invagination; fig. 1, F, final form. In fig. 1, E, the grooves are numbered in the order of their appearance. [Throughout figures—xylem represented black; phloem, white; fibres, dotted.] In this figure only principal bundles included.

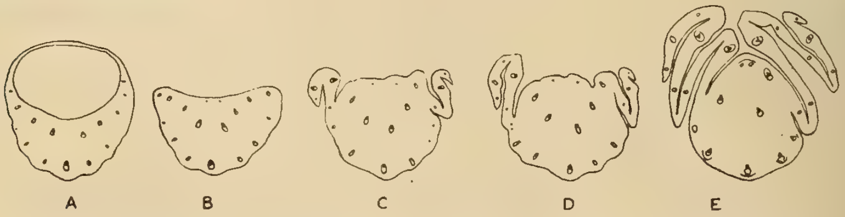

Fig. 2.—*Cocos Romanzoffiana*, Cham. Series of transverse sections from below upwards through second foliage leaf of seedling (×14). Fig. 2, A, sheath; fig. 2, B, petiole; fig. 2, C–E, stages in invagination.

That development proceeds on the same general principle in both Feather- and Fan-palms, is demonstrated by the comparison of fig. 1, A–F, with fig. 2, A–E, drawn from the second foliage leaf of *Cocos Romanzoffiana*. This series does not reach high enough into the leaf to show the final form of the limb, but it may be supplemented by reference to figs. 3, A–D, which were drawn from a set of sections cut from the third plumular leaf of another Feather-palm, *Oreodoxa regia*, H. B. et K. Fig. 3, A, shows an early stage in the grooving of the petiole; this section is interesting for comparison with the corresponding stages in the two Palms already considered, because the first grooves, in this case, are more or less parallel to the upper surface of the petiole, instead of being approximately at right angles to it.

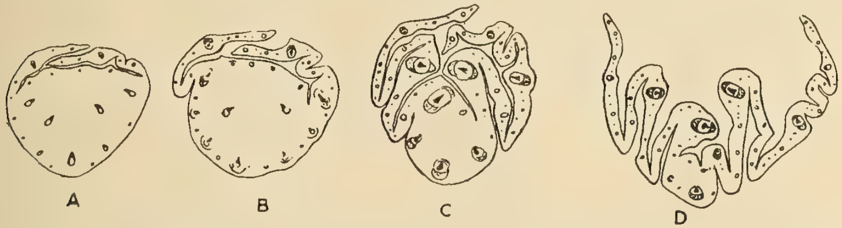

FIG. 3.—*Oreodoxa regia*, H. B. et K. Series of transverse sections through upper part of petiole, and limb, of third plumular leaf (first foliage leaf, which was preceded by two scale leaves) of seedling (×14); fig. 3, A, early stage of invagination of petiole.

As we have indicated on p. 250, two alternative explanations have hitherto been put forward to account for the resemblance of the young Palm leaf to a folded fan: (1) that there is a *splitting* of the tissues; and (2) that the rudimentary organ becomes *folded* in the course of development. I find it impossible to accept the first of these views, since my observations on the arrangement of the elements in the neighbourhood of the grooves entirely confirm the conclusion—already drawn by Hirmer* from a study of the detailed histology in certain genera—that no actual splitting takes place. But I am, on the other hand, unable to accept the second view, which Hirmer himself supports, namely, that the apparent plication is due to the folding of an originally simple leaf-rudiment ("Faltung der ursprünglich einfachen Blattanlage"). The word *folding* necessarily implies something which is the converse of the eventual unfolding of the blade. But no process takes place in the leaf-rudiment which can justly be described by such a term. I think it will be recognised that the way in which the grooves penetrate between the vascular strands, as shown in figs. 1–3, puts the folding hypothesis entirely out of court. My observations thus fail to support either the splitting or the folding interpretations; but they have led me to a third view, namely, *that the plications originate by invagination*, or, in other words, by differential growth in the course of which the outer

* Hirmer, M. (1919).

cell-layers develop more rapidly than the inner tissues, and consequently, become wrinkled, or folded in, along certain lines. In relation to the epidermis alone, the word "folded" might perhaps be admissible, but the extension of the term to the leaf as a whole cannot be justified. It may possibly be thought that I am exaggerating the value of a slight distinction, in laying so much stress upon the difference between invagination and folding. But this distinction is by no means so trivial as it may appear at a casual glance, and I believe, moreover, that the impression conveyed by the description of the young Palm leaf as "folded" has been just sufficiently inexact to prevent botanists realising the true morphological nature of the leaves of Palms—a subject to which we shall return in a later section of this paper (p. 260).

When we come to analyse the process of invagination more closely, we see that it must depend, in the last resort, on a tendency to disproportionate rapidity of growth in the surface layers as compared with those that are more deep-seated. And that such disparity should exist in the leaves is not surprising, when we realise that the Palms have undoubtedly a general tendency towards hypertrophy of the superficial tissues of their various organs. The results of this tendency are witnessed in the non-vascular spines which many Palms develop on the leaf-rachis, and in the squamiform ovarial hairs, which, in such cases as *Raphia Ruffia*, Mart., form eventually a woody coat to the fruit. The comparatively large size of the root-cap in the Palms may also be a case in point, for the calyptrogen, in the Monocotyledonous root, is the only tissue which is strictly epidermal. Possibly the integumental outgrowths, which ruminate the endosperm in many genera, may be regarded as another expression of the same tendency, while a further example is afforded by the proliferations producing the "coiffe" of the young leaf—a structure which we shall consider in the following paragraph.

2. *The Membrane* ("*Coiffe*").

It has long been known that the lamellæ of the very young leaves of certain Palms are connected by a kind of ephemeral membrane—the "Hülle," "Haut," "coiffe," or "pellicule"—so that the limb resembles a closed fan sheathed in tissue paper. This membrane is ruptured when the leaf unfolds. The view taken as to the nature of this envelope necessarily depends upon that held regarding the origin of the plications. To Von Mohl, Trécul and Naumann, the membrane was a primary constituent of the leaf, and represented the survival of the superficial tissues which were not involved in the splitting which these authors postulated. By those, on the other hand, who believe that the plications originate by folding, the membrane is held to be a secondary product, since it does not penetrate into the grooves. Deinega and Hirmer, who applied microtome technique to the problem of the origin of this membrane, conclude that it arises through secondary fusions occurring at the angles of the folds of the plicate leaf. My observations are entirely in harmony with this view, which was also indicated many years ago by Eichler. In some serial sections of the plumular leaves of *Trachycarpus Fortunei* and *Thrinax excelsa*, cut by Miss Ethel Sargant, I have been able to observe proliferated epidermal cells occluding the mouths of the invaginations (fig. 4, A–C). This occluding tissue no doubt represents the delicate early stage of the membrane which, later on, becomes so conspicuous a feature of the young leaf. The condition observed in these two genera thus confirms Hirmer's view, which was based on a study of another genus—*Phœnix.* It is much to be desired that some botanist, working at a tropical station where the leaves of a number of growing Palms are available, would give us a comprehensive account of the structure and history of the "coiffe."

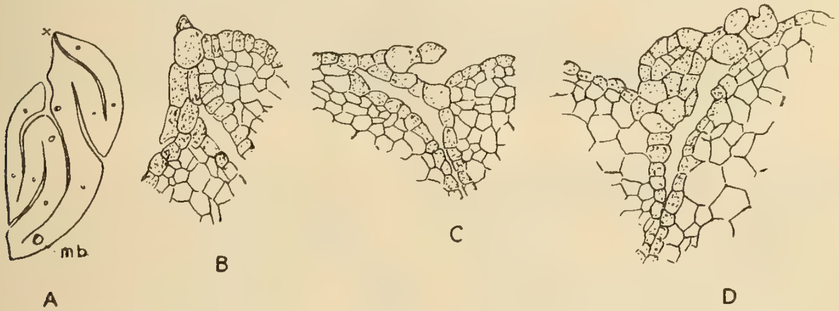

FIG. 4.—Epidermal proliferations in seedling leaves, from sections cut by Miss Ethel Sargant. Fig. 4, A–C, *Thrinax excelsa*, Lodd. Fig. 4, A, transverse section of third plumular leaf (×47); *m.b.*, median bundle. Fig. 4, B, mouth of invagination marked × in fig. 4, A (×318). The epidermis and its proliferation, which occludes the opening of the groove, are dotted. Fig, 4, C, another occluded groove, from lower down in the same leaf (×318). Fig. 4, D, *Chamærops Fortunei*, Wendl. Mouth of invagination occluded by epidermal outgrowth, from a transverse section similar to that shown in fig. 1, F (×318).

3. *The Terminal "Gland."*

Attention was drawn by Baillon* in 1895 to the fact that the first leaves produced by a Palm seedling are usually entire and parallel-nerved, and end in "une sorte de glande terminale, appareil excréteur, dont l'existence est passagère." I have observed this apical structure in the plumular leaves of *Phœnix dactylifera* (fig. 5, A–C) and *Pritchardia filifera* (fig. 5, D–F). In these cases the main part of the leaf-limb is, as usual, dorsiventral, whereas the

* Baillon, H. (1895).

apex is solid, and cylindrical in section (fig. 5, C and F). I have seen evidence of the glandular function which Baillon attributes to this apical region in the case of *Bentinckia nicobarica,* where the tip of a plumular leaf bore conspicuous stomates and contained a plexus of tracheids. After cutting successive sections of the distal region of these leaves, I have come to the conclusion that the solid tip cannot be treated as if it were a separate organ, for it appears to owe its existence merely to the fact that the invaginations die out before they reach the distal end of the leaf. In *Pritchardia filifera* the solid apical part of the leaf is, as it were, slightly undercut by the termination of the median invagination, so that a minutely hooded apex is produced (fig. 5, E). The solid tips of the seedling leaves of *Pritchardia* and *Phœnix* seem to me exactly comparable with that of such a leaf as *Crocus,** in which, also, the form of the apex is the result of the failure of the grooves to reach to the extreme end.

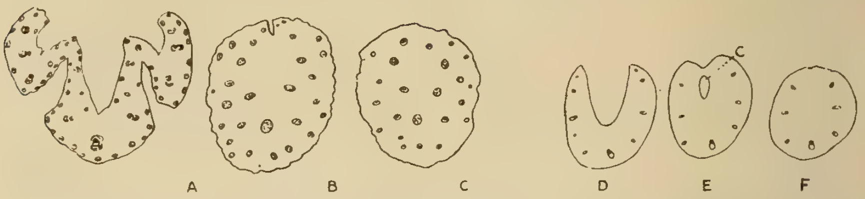

Fig. 5.—Solid tips of plumular leaves. Fig. 5, A–C, *Phœnix dactylifera*, L. Series of transverse sections from below upwards through apex of first foliage leaf to show dying out of invaginations (×14); bundles consist chiefly of fibres. Fig. 5, D–E, *Pritchardia filifera*, Lind. Series of transverse sections through apex of first foliage leaf (× 14); fig. 5, E, shows that the apex is slightly hooded; *c*, cavity.

(4) *The "Ligule" and "Dorsal Scale."*

The origin of the outgrowths which occur at the base of the limb in the Fan-palms, and are commonly known as the "ligule" and "dorsal scale," cannot be studied satisfactorily in seedlings, since it is only in the later leaves that they arrive at their full development. The "dorsal scale" is seldom conspicuous, but the "ligule," though it is very variable in size and shape in different species, is often a striking object; fig. 6 shows its appearance in the case of the mature leaf of *Trachycarpus excelsus.* In order to follow the developmental history of these structures, I cut serial transverse sections through the apical bud of a well-grown shoot of *Rhapis humilis.* Fig. 7, A–D, represent the transition from stalk to limb in one of the young leaves of this bud. It will be seen that the invaginations, which are responsible for the

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* Arber, A. (1921), fig. 57, B, C, p. 324.

"plication" of the limb, fail, at their proximal limit, to reach the epidermis either on the ventral or dorsal side, so that part of the petiolar surface is, in each case, left overarching the base of the limb. This produces the "dorsal scale" at the back (fig. 7, B, *d.c.*), and the more prominent "ligule" on the ventral side (fig. 7, C, D, *v.c.*). The hooded tip of the plumular leaf of *Pritchardia filifera* (fig. 5, E) is a comparable case on a smaller scale, though here it is the distal end of the median invagination which is overarched by the ventral surface of the limb. I think it will be agreed that the almost cup-like form produced in *Trachycarpus excelsus* by the base of the limb and the "ligule" together (fig. 6), is consistent with the view that the "ligule" merely represents the limiting region of the uninvaginated proximal part of the petiole.

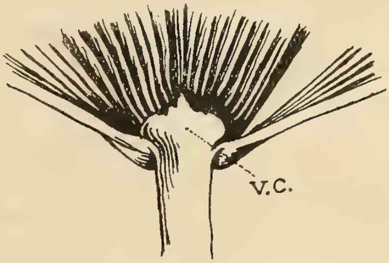

Fig. 6.—*Trachycarpus excelsus*, H. Wendl. Ventral view of junction of leaf-limb and petiole to show "ligule" or ventral crest, *v.c.* ($\times \frac{1}{2}$).

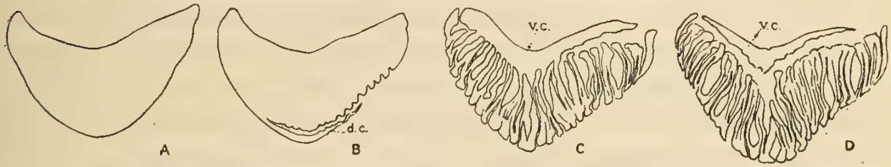

Fig. 7.—*Rhapis humulis*, Blume. Series of transverse sections through young leaf of mature plant, passing through junction of petiole and leaf-limb from below upwards (×9, circa); fig. 7, A, top of petiole; in fig. 7, B, invagination is just beginning and the slightly developed "dorsal scale" or dorsal crest (*d.c.*) is distinguishable; in fig. 7, C, the "ligule" or ventral crest (*v.c.*) is just becoming detached, while in fig. 7, D, it is entirely free. The asymmetry of fig. 7, B and C, is due to a slight obliquity in the series of sections.

The bearing of the interpretation of the "ligule" and "dorsal scale," which I have just outlined, upon the homologies of the Palm leaf, will be considered in the next section of this paper.

III. *The Morphological Nature of the Palm Leaf.*

There has never been any question among botanists as to the nature of the sheath which forms the extreme base of the Palm leaf; its homology with other leaf-sheaths is obvious. In certain Feather-palms (*Desmoncus, Calamus*) the apex of the sheath is continued upwards into the so-called ochrea, which clearly corresponds to the tubular ochrea of the Polygonaceæ, etc.

The sheath is succeeded by a stalk, for which I have throughout this paper used the word "petiole," but of which the homologies, at least in the case of the Fan-palms, are a matter of controversy. Domin* takes the view that the "ligule" of the Fan-palms is indeed a true ligule, corresponding to that of the Gramineæ, etc., and to the ochrea of the Rotangs; and since ligules are always defined as belonging to the leaf-sheath region, he draws the conclusion that in the Fan-palms the leaf-stalk, which comes below the "ligule," cannot be a true petiole, but must be of leaf-sheath nature. This view cannot but seem strained and artificial when one realises that it consigns the leaf-stalks of Fan- and certain Feather-palms to different morphological categories. Glück,† who also regards the ventral outgrowth as ligular, steers a middle course by treating it as the free apex of an extremely elongated ligule, which is fused with the petiole as far as the point of junction of stalk and limb. This speculation is ingenious, but, as I hope to show, unnecessary.

Both Domin's and Glück's views as to the nature of the "petiole" in the Fan-palms, stand or fall with the question of the homologies of the "ligule"—a problem which we must now consider. I should like to point out, in the first place, that it seems to me that any theory of the "ligule" must also take account of the corresponding structure which often occurs at the back of the leaf, and is known as the "dorsal scale." The latter is more variable than the "ligule," and though in some Fan-palms it is scarcely developed at all, I have observed in a Palm, grown under the name of *Sabal filamentosa,* that it may reach much the same degree of conspicuousness as the ventral structure. An inspection of the leaves of this Palm certainly suggests that no explanation of the origin of the "ligule" can be accepted which does not also embrace the "dorsal scale." But it is clear that the "dorsal scale" cannot be ligular, since no ligule is ever located *behind* the leaf to which it belongs, and it seems to me highly improbable that the ventral outgrowth should belong to a category from which the corresponding dorsal outgrowth is necessarily excluded.

In a preceding section of this paper I have discussed the ontogeny of the "ligule" and "dorsal scale," and the evidence there adduced has led me to the view that these structures have no more claim to be treated as distinct organs than have the solid apice of the plumular leaves described on pp. 255–6. The invaginations of the limb, in their proximal region, burrow as it were, under the ventral surface of the petiole, and to a much slighter extent, under the dorsal surface; on the ventral side the penthouse thus produced happens to be a striking object to the eye, and it may possibly become still more noticeable by suffering some further elongation after the invagination of the limb is completed, but these are not reasons which can entitle it to rank as a morphological entity. The conclusion I have reached is that the "ligule" and "dorsal scale" belong to the petiole, and are merely the outcome of the peculiar mode of limb-development characteristic of the Fan-palms. The appearance of these structures in the Fan-palms, and their absence in the Feather-palms, is due to the fact that in the Fan-palms the whole series of invaginations start from the same level, whereas, in the Feather-palms, where the plications occur to right and left of the median rachis, there is no crowding together of the invaginations at a single point.

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* Domin, K. (1911).

† Glück, H. (1901).

My view of the morphology of the "ligule" and "dorsal scale" leads me to suggest that these terms should be dropped, since the structures in question have nothing to do either with ligules or with scale-leaves; the term ligule will then be left for the tubular ochrea of *Desmoncus*, etc., to which it rightfully belongs. The best substitutes seem to be "ventral crest" and "dorsal crest," expressions based upon the word "Crista," already used by Drude* for the "ligule." If my interpretation of these structures be accepted, it leads inevitably to the conclusion that the leaf-stalk, both of the Fan- and Feather-palms, is the basal part of a true petiole.

And now, finally, we have to consider the nature of the leaf-limb. Strikingly different in aspect as are the mature blades of typical Fan- and Feather-palms, there is not, morphologically, any impassable gulf between the two types. If the median rachis is short or non-existent, we get the "fan" form, while, if there be long-continued growth, the "feather" leaf is produced; the leaves of such a genus as *Licuala*† show transitional characters. It seems possible that the "fan" type is the older, since the palmate form predominates among fossil Palms, while the Feather-palms are in the majority at the present day.‡ But the relative age of the two types must at present be treated as an open question, since they were both apparently in existence in the Upper Cretaceous. The evolution of the "feather" from the "fan" (or *vice versâ*) must have occurred more than once in the history of the family, since both types may be found among Palms which are regarded by systematists as allied. The sub-family Coryphoideæ, for instance, includes two tribes—the Phœniceæ, which are feather-leaved, and the Sabaleæ, which are fan-leaved; in the Lepidocaryineæ, again, there are two tribes differing in the same character—the fan-leaved Mauriteæ and the feather-leaved Metroxyleæ. These considerations seem to show that the various types of Palm leaf may safely be treated as homologous, whether they assume the palmate or the pinnate form.

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* Drude, O. (1889).

† Wendland, H. (1879).

‡ Unger, in Martius, K. F. P. von (1823–50).

As I have shown on pp. 251–4, the plicate limb, both in the Fan- and Feather-palms, is not, as has hitherto been commonly assumed, the result of the folding of a flat leaf-blade; it takes its origin, on the contrary, from the upper region of the leaf-stalk, through a series of invaginations which penetrate the tissues of this organ, and elaborate its more or less radial structure into a flattened and perfectly dorsiventral limb. My observations on the ontogeny thus indicate that the Palm leaf is not, as has been generally supposed, an organ whose structure is almost without a parallel, but that it falls into line with the leaves of other Monocotyledons (*e.g.*, certain Irids).*

I thus regard the Palm leaf, as a whole, as a petiolar phyllode, and its blade as a pseudo-lamina, analogous to, but not homologous with, the blade of a Dicotyledon. The fundamental identity of the leaf of the Palms with that of other Monocotyledons is, however, soon masked by secondary fusions, and, a little later, by the disintegration and tearing into segments, which the leaf undergoes in passing to its peculiar definitive form.

IV. SUMMARY.

The evidence from ontogeny and comparative morphology, brought forward in the present paper, leads to the following conclusions:—

1. The leaf-stalk, which succeeds the basal sheath, is, both in the Fan- and Feather-palms, the basal or proximal region of the true petiole.

2. The "fan" or "feather" limb is not, morphologically, a lamina, but is a modification of the distal region of the true petiole. The complex plication of the limb arises through the development of a series of invaginations which penetrate into the leaf-stalk tissue between the bundles.

3. The "ligule" and "dorsal scale" of the Fan-palms are not morphological entities, but merely represent the adaxial and abaxial distal margins of the uninvaginated proximal region of the petiole. The terms "ventral crest" and "dorsal crest" are proposed as substitutes for the terms "ligule" and "dorsal scale."

4. The Palm leaf, regarded as a whole, is, on the present interpretation, a petiolar phyllode with a pseudo-lamina—a conception which brings it into essential relation with the leaves of other Monocotyledons.

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* Arber, A. (1921).

Acknowledgments.

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Studies in the Fat Metabolism of the Timothy Grass Bacillus.

By Marjory Stephenson, Beit Memorial Research Fellow,
and Margaret Dampier Whetham.

(Communicated by Prof. F. G. Hopkins, F.R.S. Received December 14, 1921.)

(From the Biochemical Laboratory, Cambridge.)

Studies in fat metabolism have hitherto been chiefly carried out on highly specialised vertebrate tissue. By making investigations on a unicellular organism, more susceptible of laboratory control, two objects were in view: (1) to trace the stages by which the long straight chains of the fatty acid molecules are built up from the constituents of the nutritive medium; (2) to follow the circumstances and course of their subsequent breakdown. The Timothy grass bacillus was the organism selected, on account of (1) its high content of fat, and (2) its relationship to the tubercle bacillus, any facts substantiated by study of the one probably helping to throw light on the chemical habits of the other.

METHODS OF CULTURE AND ANALYSIS.

The medium employed contained inorganic salts in the following proportions:—

Potassium phosphate (K_2HPO_4)	0·1 grm.	per 100 c.c.
Magnesium sulphate ($MgSO_4 \cdot 7H_2O$)	0·07	,,	,,
Ammonium phosphate (Am_2HPO_4)	0·4	,,	,,
Calcium carbonate	excess.		

together with traces (about 0·006 grm. per 100 cubic centimetres) of sodium chloride, introduced when the organism was sown. The calcium carbonate was used to maintain the P_H of the medium at a constant value, namely, about 8·0. The ammonium salt formed the only source of nitrogen. The source of carbon varied with different experiments and will be discussed later; nothing more complex than glucose was employed.

Various types of culture vessel were tried, but the only one with which we were able to attain any degree of success was the Roux bottle. Attempts were made to use flasks or large bottles, with rubber stoppers fitted with delivery tubes through which samples of the medium could be withdrawn at intervals. Numerous attempts all proved completely unsuccessful for the following reasons:—(1) The organism grows mainly in a scum on the surface of the medium. If a deep vessel is employed this scum is far removed from the layer of chalk on the bottom, which consequently fails in its function. For this or other reasons connected with aeration, the growth of the organism slows down and comes to a standstill long before the organic constituent of the medium is used up. (2) The withdrawal of samples by means of delivery tubes plugged with cotton-wool proved completely unsuccessful. When the medium was shaken in order to distribute the growth evenly before taking a sample, the scum formed clots or flakes which tended to float, making it impossible to blow out a fair sample of medium through the delivery tube.

The method finally adopted was identical with that first employed, viz., culture in Roux bottles. The bottles, each containing about 5 grm. of calcium carbonate, were plugged in the usual way and baked for an hour at 130°–140°. Exactly 150 c.c. of medium were then introduced into each bottle, and the set of bottles was steam-sterilized on each of three successive days.

The culture was first sown on glycerol potato slopes and incubated for four to seven days. A number of test-tubes (6 inch by 1 inch) containing a small quantity of clean sand, together with about 5 c.c. of saline, were plugged and autoclaved. Into one of these the growth from the potato slopes was removed by a platinum loop. The growth from one potato slope was sufficient for two or three Roux bottles. When sufficient growth had been removed to sow the required number of bottles, it was emulsified.

The emulsification of this organism presents difficulties. The apparatus shown in fig. 1 was employed. A is a solid glass club, the handle of which passes through tubes B and C. These are arranged to form a mercury seal. The rubber stopper D is fitted into a test-tube of the same size as those containing the sterile sand and saline, and the whole is wrapped in paper and sterilized in an oven at 120°–130° for an hour. Stopper D is then removed from the empty tube. At the same moment the cotton-wool plug is removed from the tube containing the organism, sand and saline, and this tube

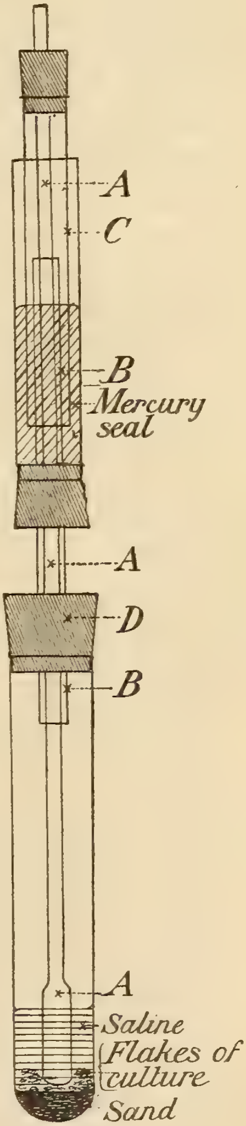


Fig. 1.

immediately replaces the empty one, and is closed by stopper D. Mercury (kept under 5 per cent. formalin) is then introduced by means of a sterile curved pipette to form a seal and prevent contamination of the tube whilst allowing sufficient play to the glass rod. The pieces of growth can be ground in the sand and a fairly even emulsion formed. When this is complete, the club and seal are withdrawn with the stopper D and the cotton-wool plug is replaced in the test-tube, after about 25 c.c. of sterile saline have been introduced and mixed with the emulsion. To avoid sowing a contaminated culture, the diluted organism was then incubated for 24 hours and sown on to slopes of tryptic agar, which medium favours the rapid growth of contaminating organisms. After 24 hours' incubation, the growth on these slopes was examined by the Ziel-Neelsen method of staining. If satisfactory, the original emulsion was sown by means of sterile pipettes, 1 c.c. to each Roux bottle. We have employed this method for a large number of experiments and have only once suffered from contamination, which was detected on the slopes before the bottles were sown.

The bottles thus sown were incubated at 37°, and sample bottles were withdrawn at intervals in order to analyse both the medium and the organism grown upon it. It will be seen that the success of the experiment depends on introducing into each bottle an equal amount of the emulsified culture. The analysis of duplicate Roux bottles withdrawn simultaneously shows agreement within the limits of accuracy of the analytical methods used, as will be shown later.

Two methods are most frequently used to estimate the amount of growth of any organism: (1) plating out and counting colonies; (2) separation of the growth by centrifuging, washing, drying, and weighing direct. The former was impossible in our experiments owing to the agglutinated character of the growth; the latter was equally unsuitable, since the growth was always mixed with the powdered calcium carbonate employed to control the reaction of the medium. In our experiments, therefore, we regarded the protein nitrogen synthesized as a measure of the growth of the organism. This protein nitrogen was precipitated by colloidal iron and estimated by Kjeldahl's method. The other characteristic of the organism to be examined was its fat content. The lipoid material of the bacillus may be conveniently divided into two: fraction A is that extracted from the dried organism by purified ether; this fraction contains no phosphorus. From the residue after extraction of A is obtained fraction B, by treatment with boiling alcohol and a subsequent second extraction with ether; B contains phosphorus. For convenience fraction A has been termed the "fat" fraction, B the "phosphatide" fraction, and the sum of the two "total lipoid."

Estimation of Synthesised Protein.

The contents of one Roux bottle were washed quantitatively into a beaker and made slightly acid with acetic acid; 20 c.c. of "dialysed iron" were added and the whole heated on a water-bath, filtered whilst hot, and washed with hot water till free from phosphate. The precipitate and paper were then transferred to a Kjeldahl incinerating flask and the nitrogen estimated in the usual manner. The results are calculated to represent grm. of nitrogen in 100 c.c. medium, *i.e.*, two-thirds of one Roux bottle.

Estimation of Lipoids of Bacillus.

(1) "*Fat.*"—The contents of one Roux bottle (150 c.c.) were transferred quantitatively to a 250-c.c. measuring flask, and made up to volume. They were then filtered through a dry pleated filter into a dry flask, and the filtrate was set aside for estimation of the constituents of the medium. The precipitate, consisting of the insoluble constituents of the medium together with the agglutinated bacteria, was transferred on the filter paper to a dish and dried in an atmosphere of nitrogen at about 97°. The final stages of the drying were carried out *in vacuo*. The filter paper and dried contents were then transferred to a Soxhlet extractor and extracted with ether (purified from alcohol and aldehydic substances). The extraction flasks employed, of about 150 c.c. capacity and about 35 grm. in weight, were fitted to the extractor with ground glass joints, in order to get good quantitative results. After about 8 hours' extraction, the ether was distilled off and the flask dried in nitrogen and later *in vacuo* at 97°. The extract thus obtained represents the "fat" fraction described above.

(2) "*Phosphatide.*"—The Soxhlet thimble and contents were then transferred to a wide-mouthed extraction flask fitted with a condenser. To this about 30 c.c. of alcohol (previously distilled from caustic soda) were added; the alcohol was boiled on a water-bath for 20 minutes and then distilled off and the flask and contents dried in an atmosphere of nitrogen. The small amount of material which had dissolved out of the thimble was then washed back with ether and the thimble and contents were returned to the Soxhlet extractor; a second extraction was then made with ether and the flask dried and weighed as before. This extract is the "phosphatide" fraction referred to above.

No detailed chemical examination of these fractions has been made, but it has been shown that the "fat" fraction contains no phosphorus, whilst the "phosphatide" fraction contains a small but fairly constant quantity (estimated by Neuman's method). Both fractions were tested for nitrogen by Kjeldahl's method: traces (below 1 per cent.) were found in both fractions.

Phosphorus Estimation.

Weight of "phosphatide" fraction taken; in grm.	Mgrm. of phosphorus found.	Percentage of phosphorus.
0·0280	0·346	1·23
0·0284	0·382	1·34
0·0240	0·270	1·12
0·0241	0·270	1·12

Nitrogen Estimation.

Weight of lipoid taken; in grm.	Mgrm. of nitrogen found.	Percentage of nitrogen.
0·1126 ("fat")	0·989	0·78
0·1260 ("phosphatide")	0·823	0·73

Probably both fractions are complex mixtures of lipoid bodies similar to those obtained from the tubercle bacillus [Goris, Goris and Liot, 1920 (1)]. The two are treated separately in this research on account of the absence of phosphorus in fraction A and its presence in fraction B.

Analysis of the Medium.

As described above, the contents of one Roux bottle (150 c.c. of original medium) were made up to 250 c.c., from which the agglutinated bacteria and solid mineral matter (chalk, etc.), were then removed by filtration. The filtrate was then analysed for glucose or other carbon compound present. Glucose estimations were carried out on 5 c.c. of the diluted medium by the method of Wood-Ost [Wood and Berry, 1903, (4)]. Ammonia was estimated in the first and last samples by the method of Van Slyke [1911, (3)], in order to ensure that growth of the bacteria had not ceased through lack of nitrogen.

Experiment 1.—*Growth of the Bacillus in respect of Total Nitrogen and Lipoids on the usual Inorganic Medium, containing as well* 1 *per cent. Glucose and* 1 *per cent. Acetic Acid, added as Sodium Acetate.*

Twenty-five Roux bottles, prepared and inoculated as described above, were incubated at 37° and bottles were withdrawn at intervals in sets of two, one for the estimation of total nitrogen, and the other for the estimation of lipoids and of the organic constituents of the medium. The disappearance of acetic acid was not followed stage by stage in this first experiment, but it was ascertained that by the time the glucose had been utilised the acetic acid had also disappeared.

The reaction of the medium was kept constant by chalk, which was spread over the bottom of the Roux bottles; the P_H at the beginning, end, and all intermediate stages remained 8·0. This was tested by phenol red, and kindly checked for us by the hydrogen electrode by Miss Jordan Lloyd.

The figures given in column 1 of the following table represent the actual amounts obtained from single Roux bottles (150 c.c. each), which were of necessity the units employed; column II represents the same amounts calculated for 100 c.c. medium, from which the points on Curve 1 are taken. Where estimations were made on duplicate Roux bottles, the figures are bracketed.

Fat Synthesised.

Day of experiment.	"Fat" fraction. Weight in grm.		"Phosphatide" fraction. Weight in grm.		Total lipoids. Weight in grm.	
	I.	II.	I.	II.	I.	II.
7*	0·0054	0·0036	0·0083	0·0054	0·0137	0·0090
14	0·0198	0·0132	0·0181	0·0120	0·0379	0·0250
21	0·0399 0·0348	0·0288	0·0284 0·0280	0·0188	0·0623 0·0625	0·0420
22	Organic constituents of the medium completely utilised.					
28	0·0179 0·0268	0·0149	0·0240 0·0241	0·0160	0·0419 0·0501	0·0310
36	0·0145	0·0096	0·0230	0·0152	0·0375	0·0124
60	0·0089 0·0095	0·0061	0·0162 0·0152	0·0105	0·0251 0·0247	0·0170

* The results obtained on the seventh day are too small relatively to the experimental error to be important. They merely serve to indicate that growth had begun.

Protein Nitrogen Synthesised.

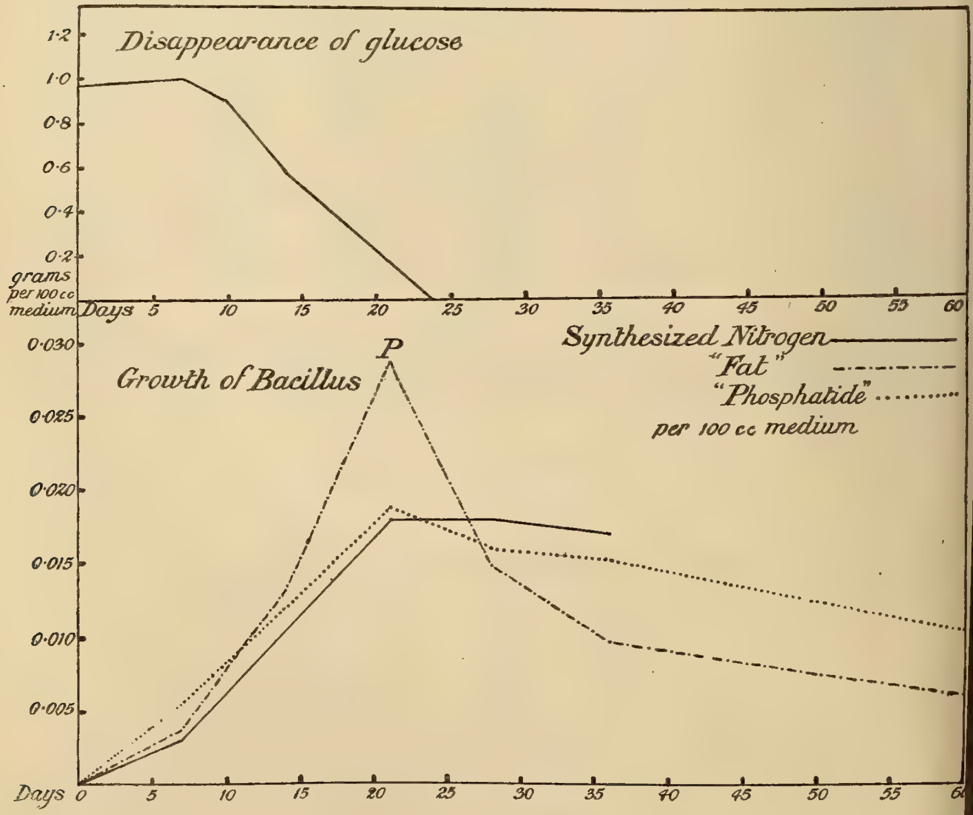
Day of experiment.	Grm. per Roux bottle. I.	Grm. per 100 c.c. medium. II.
7	0·005	0·003
21	0·027 0·027	0·018
28	0·027	0·018
36	0·026	0·017

Glucose Utilised.

Day of experiment.	Glucose present; grm. in 100 c.c. medium.	Glucose utilised; grm. in 100 c.c. medium.
0	0·97	0·00
7	1·00	0·00
10	0·90	0·07
14	0·58	0·39
21	0·14	0·83
28	0·00	0·97

NOTE.—On the 28th day the acetic acid also had completely disappeared.

It is apparent from Curve 1 that the growth of the bacillus, as measured by protein nitrogen and lipoids synthesised, reaches a maximum at the point when glucose and acetate disappear from the medium. From this point onwards, the bacillus utilises its own lipoid material, which rapidly decreases. It is particularly noteworthy that the "fat" fraction decreases at first more



CURVE 1.

rapidly than the "phosphatide" fraction, until an equilibrium is established in which the "phosphatide" fraction is maintained at the higher value. This suggests that the "fat" fraction represents a form of stored food material which is drawn upon when external sources of carbonaceous food material fail, whilst the "phosphatide" fraction, which decreases less rapidly, is probably partially composed of some unit essential to the chemical structure of the cell. The duplicate determinations of the "fat" fraction on the twenty-eighth day show a wide variation. This is in the period of rapid utilisation of stored fat. A slight retardation of the point at which maximum growth was attained and utilisation of fat began would result in a large observable difference at this point.

The results of starvation thus show that the fat metabolism of the higher animals has its prototype in this micro-organism.

It also appears from the curve that the protein nitrogen decreases very slightly during the first fourteen days of starvation, the drop between the twenty-eighth and thirty-sixth day being actually within the experimental error. The bacillus thus seems to preserve its protein material intact. It must be remembered, however, that throughout the experiment ammonia was present in the medium; the bacillus may therefore have broken down cell protein as well as lipoid material, and have reconstituted the former from the ammonia of the medium and the fatty acid chains of the lipoids. Unfortunately no material was available for a final protein nitrogen estimation on the sixtieth day.

It will be noticed that there is a small but definite growth of bacillus on the seventh day of the experiment, while the glucose is apparently untouched. We have confirmed this observation repeatedly in experiments where glucose forms the only organic food material. It may have its explanation in the small amount of food material carried over from the potato slopes, or it may be that in the initial breakdown of the sugar molecule a small quantity of a reducing body accumulates which is erroneously estimated as glucose; such a body has been searched for but not as yet found.

During the course of the foregoing experiment, in 100 c.c. of medium, approximately 1 grm. of glucose and 1 grm. of acetic acid (as acetate) disappeared, together with certain unascertained quantities of mineral material and ammonia. Their place was taken by 0·018 grm. of protein nitrogen, corresponding roughly to 0·1 grm. protein, and 0·041 grm. of mixed lipoid material.

In a similar experiment in which glucose was the sole organic constituent of the medium, 1 grm. of glucose gave rise to 0·0189 grm. of protein nitrogen and 0·028 grm. of lipoid material. In an experiment such as the latter, it was hoped that some intermediate products in the change from glucose to fat might be isolated, and experiments with that end in view were made.

Experiment 2.—*Search for Intermediate Products.*

Five Roux bottles, in all 750 c.c. of medium, containing the usual inorganic salts, with calcium carbonate and 1 per cent. glucose, were inoculated in the usual way and incubated for fourteen days. The total contents of the five bottles were made up to 2,000 c.c., and filtered. The glucose was estimated in the filtrate and was found to be 0·11 per cent. (calculated on the original medium). 0·89 per cent. glucose had thus disappeared from the original 1 per cent. The medium still had its original reaction, P_H 8. Half the filtrate (corresponding to 375 c.c. of the original medium) was steam-distilled, first from neutral solution. The distillate was collected in cold water in fractions and examined for iodoform-producing bodies; a slight iodoform reaction was obtained from the distillate collected during the first five minutes, but none from subsequent fractions. The iodoform could only be detected by the smell; no visible precipitate was obtained.

The residue in the distilling flask was then acidified with phosphoric acid and again steam-distilled, the distillate being collected in standard 0·1N baryta (50 c.c.). At the end of 1½ hours the distillation was stopped and the baryta back-titrated with hydrochloric acid, using phenol phthalein. The difference gave the acidity due to volatile acids and carbonic acid, of which a considerable quantity had come over. The carbonate was then filtered off, washed, transferred to a flask, mixed with 50 c.c. 0·1N HCl, and boiled under a reflux condenser fitting into the neck of the flask. The residual acid was back-titrated with baryta. The results from 375 c.c. of the original medium were as follows:—

Carbonate and volatile acids	36·2 c.c.	0·1N baryta.
Carbonate alone	29·4 ,,	0·1N ,,
Volatile acids	6·8 ,,	0·1N ,,

Calculated as acetic acid this is equivalent to 0·011 per cent. acetic acid in the original medium. The residue from the steam-distillation of the total medium (750 c.c.) was evaporated on a water-bath to about 150 c.c., filtered, and the filtrate extracted with ether in a continuous extractor for 24 hours. The solid matter was dried and extracted in a Soxhlet apparatus. The ethereal extract from the liquid extraction contained no solid organic acids. The aqueous residue left after the evaporation of the ether was tested for lactic acid by Hopkins' thiophene test; lactic acid appeared to be present in traces, but this could not be established with certainty owing to the rapid darkening of the solution by easily charred matter. No solid material was obtained from the Soxhlet extractor.

These results were confirmed by two other experiments, slight variations only being obtained in the amount of volatile acids distilled over. It seemed therefore that, under the conditions prevailing in these experiments, intermediate products in the breakdown of glucose accumulate only in such traces that attempts at identification are hopeless.

Experiment 3.—*An Attempt to Obtain Fermentation apart from the Growth of the Organism.*

Six Roux bottles were filled with a medium containing the usual inorganic salts and 1 per cent. glucose. From six otherwise exactly similar bottles the ammonium phosphate was omitted. These twelve bottles were sown more thickly than usual with the emulsion of the bacillus. At the end of fourteeen days the sugar in the bottles containing no ammonia was estimated and found completely untouched, whilst that in the control bottles had disappeared in the normal manner. It thus appears that under these conditions fermentation does not occur apart from growth, which in its turn is dependent on a supply of nitrogen.

Direct attempts at isolation of intermediate products having failed, indirect evidence was sought by growing the organism on *possible* intermediate products in the breakdown of the sugar molecule, in order to ascertain: (1) whether the organism was capable of utilising and growing on the intermediate products; (2) if growth took place, how the formation of fat was affected.

The formation of protein nitrogen (precipitated by colloidal iron) was taken as a criterion of growth, the lipoids corresponding to the nitrogen synthesised at each period being separately estimated as previously described. At the point of maximum lipoid formation (*e.g.*, point P, curve 1), the growth of the organism was considered to have attained its maximum, and at that point the lipoid formation was compared with the protein nitrogen synthesised.

Experiment 4.—*Growth on, and Utilisation of, Lactic Acid.*

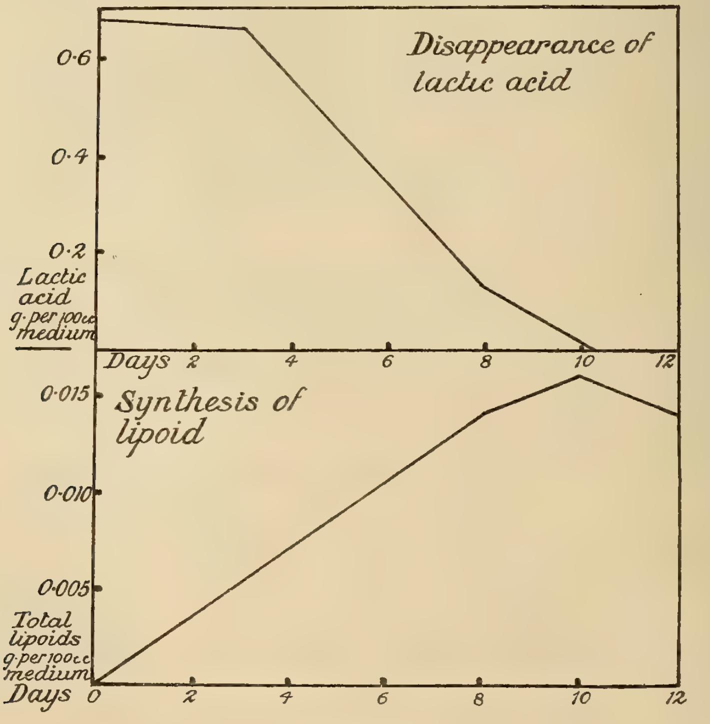
A series of Roux bottles was started in the usual way containing the usual inorganic salts and also 0·68 per cent. lactic acid (dl) added as sodium lactate. Sample bottles were withdrawn at intervals and analyses of the organism carried out as in Experiment 1. Lactic acid was estimated in

25 c.c. samples of the diluted medium by the extraction method of Hopkins and Fletcher [1907, (2)], and weighed as zinc lactate.

The disappearance of lactate from the medium and the synthesis of total lipoids is summarised below and shown graphically on Curve 2.

Day of experiment.	Lactic acid present; grm. in 100 c.c. of medium.	Lactic acid utilised; grm. in 100 c.c. of medium.	Total lipoids formed; grm. in 100 c.c. of medium.
0	0·68	0·00	0·00
3	0·66	0·02	Not estimated.
8	0·13	0·55	0·014
10	0·01	0·67	0·016
12	0·00	Total	0·014

Grm. nitrogen synthesised per 100 c.c. on 10th day, 0·018.



Curve 2.

It will be seen from the above that the organism readily used lactic acid as its sole source of organic food material, and that the course of its growth does not differ materially from that on glucose and acetic acid already established. Similar results were obtained when the lactic acid was added in the form of calcium lactate. The relationship between the total lipoids formed and the total protein nitrogen at the point of maximum growth will be discussed later.

Experiment 5.—*Growth on, and Utilisation of Acetic Acid.*

Roux bottles containing the usual inorganic salts, with 0·5 per cent. acetic acid as sodium acetate, were steam sterilised and sown in the usual way.

Estimation of the Acetic Acid.—The contents of one Roux bottle (150 c.c. of original medium) were made up exactly to 250 c.c., filtered through a dry filter into a dry flask, and the acetic acid then estimated by distillation of 20 c.c. acidified with phosphoric acid. The usual method of estimating acetic acid by steam distillation was found unsatisfactory. The amount of distillate which must be collected before the acetic acid has completely come over is at least 2,000 c.c.; this contains large and variable quantities of carbon dioxide, which must be separately estimated by collecting the distillate in excess standard baryta, neutralising the excess with standard hydrochloric acid, filtering off and separately estimating the barium carbonate. The whole process takes between three and four hours, and gives moreover very variable and unreliable results. Instead, a modified procedure was adopted.

The distillation of the acetic acid was carried out *in vacuo.* The apparatus used was identical with that described by Van Slyke (*loc. cit.*) for the estimation of ammonia, except that in place of the second smaller distilling flask, a thick-walled filtering flask was found more convenient; 10 c.c. of diluted medium (or an amount containing from 0·06 to 0·1 grm. of acetic acid) was placed in a Claissen flask of 300–400 c.c. capacity, fitted with a capillary tube in the first neck, and a tap in the second neck. The liquid was acidified with four or five drops of saturated phosphoric acid. An equal volume of 95 per cent. alcohol (previously distilled from caustic soda) was added. An exact volume (about 25 c.c.) of 0·1N baryta was run from a burette into the distilling flask and the filtering flask, and the capillary tube was adjusted to dip beneath the surface of the liquid. The contents of the Claissen flask were then distilled *in vacuo* (10–15 mm.), and the distillate collected in the baryta in the cooled receiver, any escape of acetic acid being prevented by the baryta in the filtering flask. The distillation was continued until only about 1 c.c. of liquid remained in the Claissen flask. The temperature during the last five minutes should be at least 60°. When the distillation was complete, air was admitted by the capillary and by opening the tap in the second neck of the Claissen flask; 20 c.c. of 50 per cent. alcohol were then poured into the flask and the distillation repeated as before, care being taken that the temperature of the water-bath reached 60° for at least five minutes. The apparatus was then disconnected, the baryta carefully washed into the filtering flask, and the excess titrated with standard hydrochloric acid. A small and variable amount of barium carbonate was always present and was separately estimated. The neutralised liquid was filtered, the solid (barium carbonate) washed twice and then transferred into a flask fitted with a small reflux condenser (preferably ground in); 10 c.c. of 0·1N HCl was then added to the contents of the flask and the whole boiled for five minutes under the reflux condenser. The excess HCl was back-titrated with baryta.

The method was tested as follows:—

10 c.c. of a solution of acetic acid were titrated direct against 0·1N baryta, using phenol phthalein; 10 c.c. of the same solution of acetic acid were distilled into baryta as described above, and the results compared. The following are the figures for the distillation:—

A. C.c. acetic acid taken.	B. C.c. 0·1N $Ba(OH)_2$ taken.	C. C.c. 0·1N HCl used for back titration.	D. C.c. 0·1N $Ba(OH)_2$ neutralised by acetic acid and CO_2 [B–C].	E. C.c. 0·1N $Ba(OH)_2$ neutralised by CO_2 only.	F. C.c. 0·1N $Ba(OH)_2$ neutralised by acetic acid [D–E].	Per cent. of acetic acid in solution.
10	25·8	2·6	23·2	0·7	22·5	1·35
10	25·4	1·6	23·8	1·4	22·4	1·34

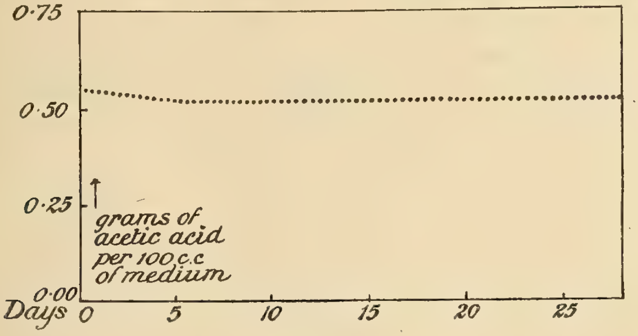
10 c.c. of acetic acid titrated direct = 22·1 c.c. 0·1N $Ba(OH)_2$.

∴ 10 c.c. acetic acid contain 0·133 grm. = 1·33 per cent. compared with 1·35 and 1·34 by the distillation method.

The disappearance of acetic acid from the medium is summarized below, and shown graphically on Curve 3.

Day of experiment.	Acetic acid present; grm. in 100 c.c. of medium.	Acetic acid utilised; grm. in 100 c.c. of medium.
0	0·53	0·00
4	0·49	0·04
28	0·46	0·07

The growth of the organism was too small to admit of accurate estimation. From this it appears that, in the conditions holding in this experiment, the organism suffers from almost complete inability to use acetic acid as a source of organic food material. The slight disappearance of acetic acid is probably due to a small amount of carbohydrate carried over from the potato slopes (see below).



Curve 3.

Since it has been shown in earlier experiments that no acetic acid accumulates during the growth of the organism on glucose, and since acetic acid has been shown by many workers to be a frequent intermediate product in the breakdown of glucose by bacteria, it seemed worth while to ascertain whether the presence of other substances might not confer on the Timothy grass bacillus the power of utilising this compound.

Experiment 5.—*Utilisation of Acetic Acid in the presence of Lactic Acid or of Glucose.*

Sixteen Roux bottles, containing the usual inorganic medium with 1 per cent. lactic acid (added in the form of calcium lactate) and 0·5 per cent. acetic acid (added as sodium acetate), were treated as described in Experiment 1. Acetic acid was estimated on 20 c.c. of the diluted medium. It was found that a correction had to be introduced owing to a small constant amount of lactic acid distilling over with the acetic acid. The amount of the correction was ascertained as follows:—20 c.c. of diluted medium, containing 1·2 per cent. of lactic acid only, were distilled exactly as described above for acetic acid. A second distillation of 20 c.c. of the medium, containing 1·2 per cent. lactic acid and 0·5 per cent. acetic acid was carried out under exactly the same conditions. The number of cubic centimetres of 0·1N baryta neutralised by the lactic acid distilled over in the first experiment was then deducted from the number of cubic centimetres of baryta neutralised by the lactic and acetic acids distilled over in the second experiment. The difference gave the number of cubic centimetres of baryta neutralised by the acetic acid only. It was found that the amount of lactic acid distilled over under the standard conditions was fairly constant (corresponding to

1·9–2·1 c.c. of baryta), and a constant deduction of 2 c.c. of baryta was therefore made.

The residue left in the Claissen flask after the acetic acid had been distilled off was quantitatively removed and the lactic acid estimated as in Experiment 4. The result was, of course, too low, owing to the loss of the lactic acid which had been distilled over with the acetic acid (*i.e.*, 0·18 grm. per 100 c.c. of diluted medium). A small addition had therefore to be made to the results obtained by extraction.

Utilisation of Lactic and Acetic Acids present together.

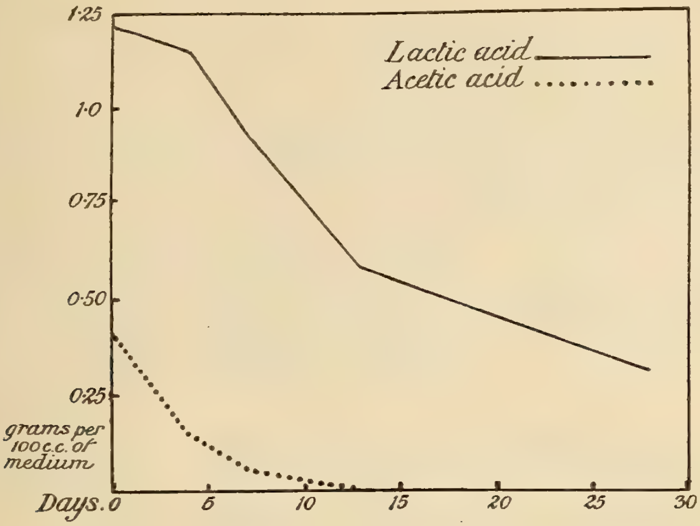
Day of experiment.	Acetic acid present; grm. per 100 c.c. of medium.	Acetic acid utilised; grm. per 100 c.c. of medium.	Lactic acid present; grm. per 100 c.c. of medium.	Lactic acid utilised; grm. per 100 c.c. of medium.
0	0·41	0·00	1·22	0·00
4	0·15	0·26	1·15	0·07
7	0·055	0·355	0·94	0·28
13	0·00	0·41	0·58	0·64
28	—	—	0·32	0·90

Two similar experiments were carried out with 1 per cent. glucose and 1·5 per cent. and 0·7 per cent. respectively of acetic acid (added as potassium acetate).

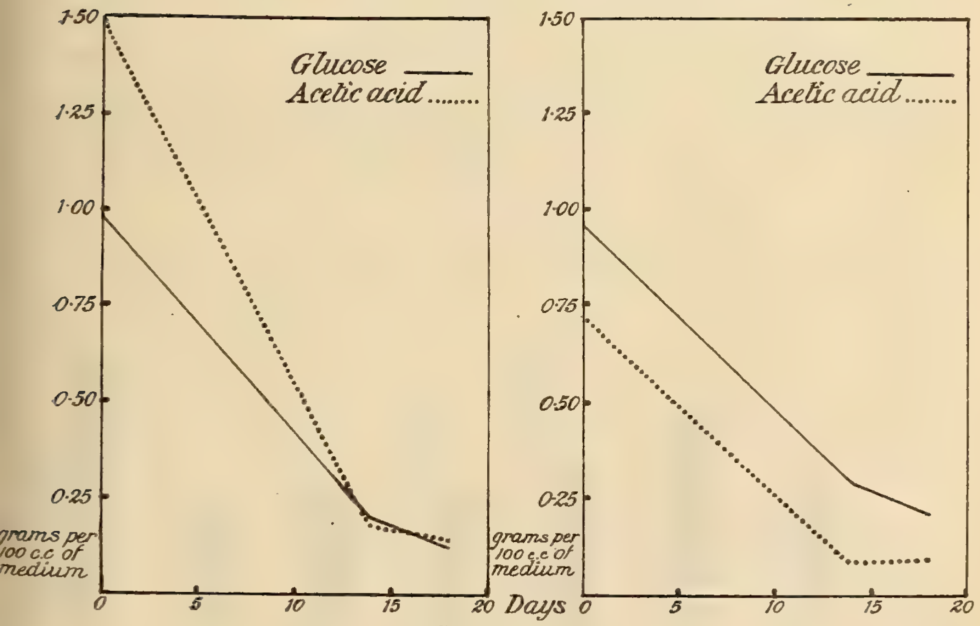
Utilisation of 1 per cent. Glucose and 1·5 per cent. Acetic Acid present together.

Day of experiment.	Acetic acid present; grm. per 100 c.c. of medium.	Acetic acid utilised; grm. per 100 c.c. of medium.	Glucose present; grm. per 100 c.c. of medium.	Glucose utilised; grm. per 100 c.c. of medium.
0	1·49	0·00	0·98	0·00
14	0·18	1·31	0·21	0·77
18	0·14	1·35	0·12	0·86
Utilisation of 1 per cent. glucose and 0·7 per cent. acetic acid present together.				
0	0·72	0·00	0·95	0·00
14	0·08	0·64	0·29	0·66
18	0·09	0·63	0·22	0·73

The above tables and accompanying graphs (Curves 4 and 5) show that, in the presence of lactic acid, or of glucose, the organism is capable of utilising the lactic acid completely.



Curve 4.



Curve 5.

Influence of Diet on the Chemical Composition of the Organism.

Having obtained growth on media with various organic constituents, it is of interest to notice what influence these substances have on the proportion of lipoids to nitrogenous material in the organism. The graph illustrating Experiment 1 shows that the lipoids and synthesised nitrogen rise to a maximum at a point closely corresponding in time to the disappearance of the organic constituents of the medium, and that after this point the lipoids decrease rapidly and the nitrogenous material very slowly. The point of maximum lipoid formation is taken as the stage at which the composition of the organism shall be estimated for purposes of comparison. The results are summarised in the following Table and shown graphically in fig. 2:—

Reference to fig. 2.	Organic constituents of medium.	Period of maximum lipoid formation in days.	Grm. in 100 c.c. of medium.		Ratio: $\frac{\text{Total lipoid}}{\text{Nitrogen}}$.
			Nitrogen synthesised by organism.	Total lipoids formed by organism.	
1	0·68 p.c. lactic acid...	10	0·019	0·016	0·84
2	1·4 p.c. lactic acid ..	11	0·026	0·020	0·78
3	1·2 p.c. „ „ .. 0·4 p.c. acetic acid ...	7	0·020	0·035	1·78
4	1 p.c. glucose	12	0·018	0·028	1·6
5	1 p.c. „ .... 1 p.c. acetic acid	21	0·018	0·042	2·34
6	1 p.c. glucose	15	0·015	0·019	1·3
7	2 p.c. „	15	0·034	0·021	0·91

The results of the two experiments made with lactic acid alone in different amounts, approximate closely in the proportion of lipoid to nitrogen (fig. 2—1 and 2). When the organism is grown on a mixture of lactic and acetic acids, no more nitrogen is synthesised than on lactic acid alone, but the lipoid is increased by about 100 per cent. (fig. 2—3). A similar effect appears when

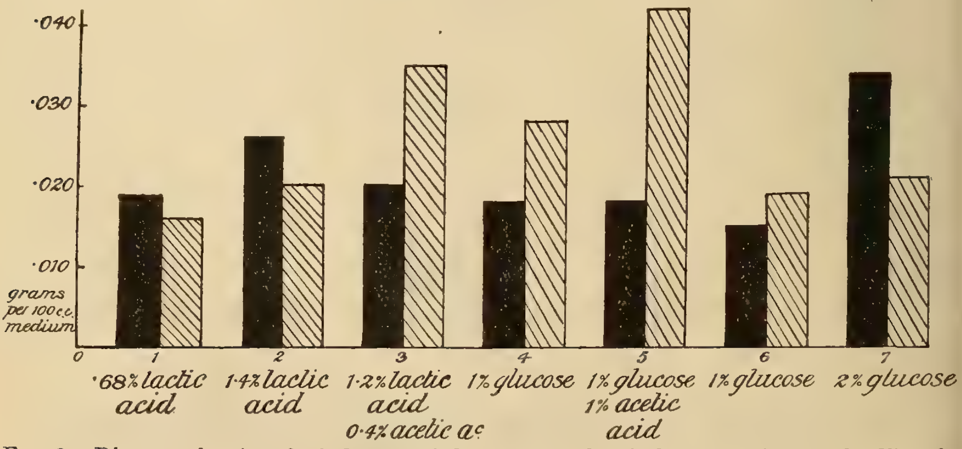


Fig. 2.—Diagram showing the influence of diet on the chemical composition of the Timothy grass bacillus.

Black blocks represent grams of nitrogen synthesised per 100 c.c. of medium. Shaded blocks represent grams of lipoid synthesised per 100 c.c. of medium.

acetic acid is added to a glucose medium (fig. 2—4 and 5); the added acetic acid does not affect the nitrogen synthesised (0·018 grm. in both cases), whilst the ratio of lipoids to nitrogen rises from 1·58 to 2·34.

It seemed possible that this result might not be due specifically to the acetic acid, but might be caused by the greater concentration of organic food stuff in the medium. Experiments made on 0·68 per cent. and 1·4 per cent. of lactic acid (fig. 2—1 and 2), and on 1·0 per cent. and 2·0 per cent. of glucose (fig. 2—6 and 7), show that this is not so. Increased concentration of food in each case results in an increased production both of synthesised protein and of lipoid material, *i.e.*, increased general growth. The production of protein is favoured rather more than that of fat, as is shown by the ratio of lipoids to nitrogen.

The inability to use acetic acid in the absence of carbohydrate or lactic acid forms at first sight an analogy to the inability of the higher animals to use fat in the absence of a minimum of carbohydrate. From this analogy, the authors were led to grow the bacillus on the sodium salts of propionic and butyric acids. Growth on both these proceeded readily in a manner comparable to growth on lactate and on glucose. The problem therefore appears to be one of some complexity. A detailed study of the growth of this organism on various simple straight-chain compounds is in progress, and will form the subject of a later communication.

SUMMARY.

The growth of the Timothy grass bacillus on a medium consisting of inorganic salts, including ammonia as the sole source of nitrogen, glucose, and sodium acetate, was followed in detail. The formation of protein nitrogen and fat (a phosphatide and a non-phosphatide fraction) were followed stage by stage, and correlated with the disappearance of glucose and acetate from the medium. Attempts were made to isolate intermediate decomposition products of glucose, but none were found.

The growth of the organism on possible intermediate products of the breakdown of glucose was then studied. The growth on lactic acid (as lactate) was very similar to that on glucose alone. The lactic acid could be completely utilised and the formation of protein and fat resembled that on a glucose medium. Growth on acetic acid (present as sodium acetate) was negligible in amount, and the acetic acid was not attacked by the organism. A modified method of estimating acetic acid is described. Growth on acetic and lactic acid (acetate and lactate) showed that the presence of lactic acid enabled the organism to utilise the acetic acid. Glucose also enabled the organism to utilise the acetic acid.

A comparison of the composition of the growth on these media showed that the acetic acid utilised in the presence of lactic acid or glucose served, not to increase the general growth of the organism, but to increase the proportion of lipoid material formed. This was shown to be a specific effect of the acetic acid, and was not merely due to greater concentration of carbonaceous food material.

Experiments were made to ascertain whether the behaviour of the bacillus on other straight-chain fatty acids resembled that on lactic acid (and glucose) or that on acetic acid. Growth on propionic acid and on butyric acid was like that on lactic acid, *i.e.*, the organism was able to grow on these compounds without the addition of other carbon compounds, and to synthesise both nitrogenous and fatty material.

The authors' thanks are due to Dr. Graham Smith for kindly providing a strain of the Timothy grass bacillus. They gladly take this opportunity of expressing their gratitude for the stimulating and encouraging criticism afforded by Prof. F. G. Hopkins during the progress of this research.

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Recoil Curves as Shown by the Hot-Wire Microphone.

By Lieut.-Colonel C. B. Heald, C.B.E., M.D., and Major W. S. Tucker, R.E., D.Sc.

(Communicated by Prof. C. S. Sherrington, P.R.S. Received November 15, 1921.)

[Plates 5–8.]

Introductory Remarks.

The subject matter of this paper deals largely with a description of a process which, in its bearing, appertains to physics rather than physiology, but as the application is entirely physiological, it has been decided to submit it as a physiological contribution.

In 1916, one of us (W. S. T.), while at work on the perfection of the hot-wire microphone, which he had invented for the location of enemy guns, realised the possibilities of the hot-wire microphone for obtaining records of the pulse, apex beat, etc., and had actually taken records both from the wrist and neck, and had shown these to members of the medical profession.

In the same year, when one of us (C. B. H.) was working upon the examination of cadets for pilots' certificates in the Royal Flying Corps, a converted "penny-in-the-slot" weighing machine was used for recording the weight. It was noticed that the machine would not permit an absolutely steady reading to be taken, as the point of the hand was in constant movement. Closer observation of this movement showed that it took place in time with the heart beat.

The obvious explanation of the movement of the hand was that the propulsion of the blood from the left ventricle of the heart towards the head during the first stage of systole was accompanied by a corresponding opposite movement of the body towards the feet. If this assumption were correct, then, knowing the weight of the body, measurement of the actual distance through which it was moved would provide a useful factor in determining the efficiency of the heart when regarded as a mechanical pump.

This simple observation with the weighing machine sufficed, however, to attract attention to the importance of employing the recoil of the body for the measurement of heart efficiency, the heart being considered as a pump.

The only previous work along these lines appears to be that undertaken by Prof. Yandell Henderson (7), who used a swinging table, in which lateral movements were prevented by an ingenious device; he was thus able to record upon a smoked drum the movements of the table multiplied by 100. His remarks on this table are interesting :—

> "With such a table, the heart beat causes not only longitudinal, but also lateral movements. The latter have not yet been examined in detail. In fact, it is necessary, in order to record the longitudinal movements with accuracy, that the lateral movements should be prevented. It is also necessary, not only that the person under examination should lie absolutely still, *but that he stop breathing during the time the heart beats are recorded.* Herein, indeed, lies the chief difficulty of the investigation, for while the total amplitude of the recoil movements is only a tenth of a millimetre, and some of the features of the curve amount to less than a tenth of this distance, respiration swings the body through a distance of many millimetres. Lastly, in order to avoid, so far as possible, errors from the table swinging back, after being moved out of plumb by the recoil, a pendulum period many times longer than the cardiac cycle was found necessary."

This author's comments, even with these precautions, showed that at least three great difficulties arose :—

(1) Periodicity in the recording apparatus.

(2) Errors due to respiratory movements.

(3) Errors due to the records having to be taken when the breath is either held, or the subject is blowing on a whistle.

He considers that if these difficulties could be overcome, our knowledge of the factors controlling the physical efficiency of the cardio-vascular system would be improved.

It appeared reasonable to think that the hot-wire microphone would give faithful records without encountering any of the difficulties above referred to, and, at the same time, provide a means of measurement and calibration.

It was realised that if such measurements and calibration could be accomplished, the results would be of value in examinations for determining physical efficiency, especially in the case of aeroplane pilots.

The recording apparatus (fig. 1) may be divided into three parts :—

(1) The microphones.

(2) The galvanometer and timing device.

(3) The photographic apparatus.

It may be here stated that some of this apparatus has already been in use in sound ranging of guns, but important modifications have been made in order to cope with the special difficulties of the method.

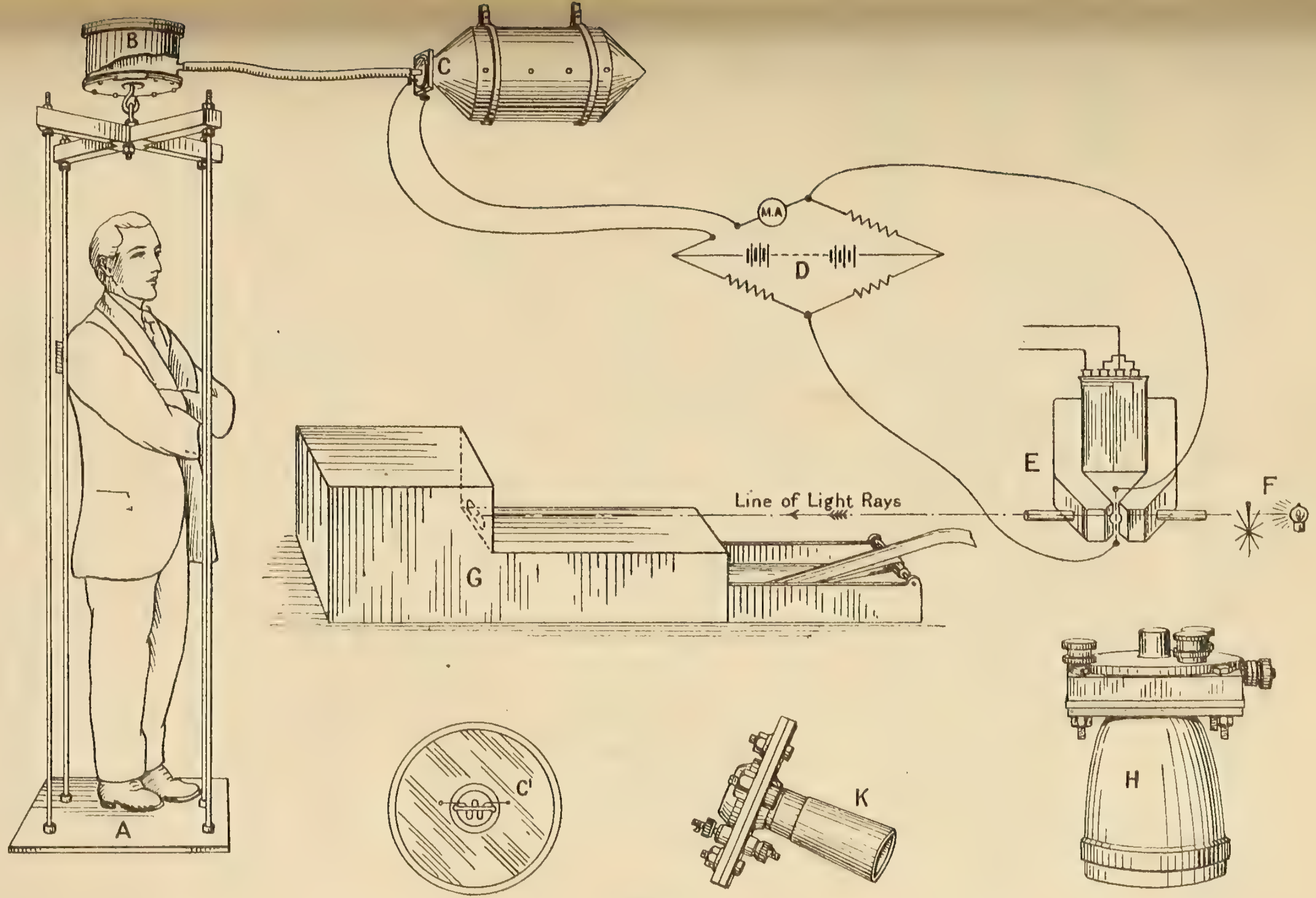


FIG. 1.

A, platform ; B, drum and diaphragm ; C, microphone and accessory cylinder ; C', microphone grid ; D, Wheatstone bridge circuit ; E, Einthoven galvanometer ; F, source of light and time wheel ; G, camera and automatic developer ; H, pulse microphone ; K, breathing microphone.

1. *The Microphone.*

The hot-wire instrument is specially capable of dealing with low-frequency vibrations such as those imparted to the human body by the heart. It consists essentially of two iron drums, connected together by a short piece of rubber tubing.

One of the drums is fixed to the ceiling of the room in which the work is done. It consists of a cylinder, the side of which is about half its diameter. The platform on which the patient is standing is supported by a hook from a diaphragm which forms the circular end of the drum. When, therefore, the heart-beats cause motion of the body, the diaphragm responds, thereby altering the pressure within the drum in a corresponding way.

A side tube, let into the wall of the drum, permits the passage of air which is set in motion as a result of these changes. This tube is connected by the rubber tube above mentioned to a second cylinder of about twice the capacity, and having conical ends, in one of which is a fitting containing the hot-wire microphone grid. The air blasts, transmitted along the rubber tube from the first drum, are passed into the second drum through a tube opening, and are projected past the hot-wire grid, thereby cooling the grid and diminishing to a corresponding extent its electrical resistance.

Records of apex beat or carotid involved the use of a second microphone, simply consisting of an open cup whose rim is of ebonite and whose base contains the microphone fitting with its grid, and the image of the "string" recording these effects is thrown on the same recording strip as the recoil curve by means of a right-angle prism. The cup is pressed with its rim against the chest or neck, and the pulse changes are indicated by air blasts passing the grid into the open air (fig. 1).

The breathing microphone (fig. 1) consists of a single strand of hot-wire, mounted on an appropriate fitting attached to a stem resembling that of an ordinary tobacco pipe, but with a much wider air channel. The mouthpiece is held in the mouth, and the temperature of the grid is simply varied by that of the air or of the breath which passes inwards or outwards.

Periodicity of the breathing is thus indicated. A very small electric current is used, just sufficient to indicate change in resistance on the galvanometer.

2. *The Galvanometer and Timing Device.*

The galvanometer employed is of the Einthoven type. Body movements are measured by deflections of a very fine wire mounted between the poles of a strong electromagnet. The instrument employed is the Souttar galvanometer. It is less sensitive than the galvanometer used with the electrocardiograph apparatus.

The galvanometer used for pulse or breathing records is of the same type as the instrument used in the Sound Ranging apparatus. It is less sensitive than the Souttar galvanometer.

All the records obtained, whether of body movement, apex beat or breathing, are obtained from one of these galvanometers acting in a Wheatstone bridge circuit.

The timing device is a rotating toothed wheel, kept in rotation by a synchronous motor, and the current which operates it is supplied from a circuit containing a contact opened and closed by a vibrating tuning fork electrically maintained. In this way, the time-wheel can only rotate at a constant speed satisfying the above conditions, and making the record in hundredths and tenths of a second.

3. *The Photographic Apparatus.*

For the production of permanent records, use was made of the automatic developing apparatus employed in gun sound ranging. This consists of a camera with a roll of sensitised paper which can be fed continuously behind a cylindrical lens; the paper thereafter passing automatically through a developing and fixing bath. The speed of the sensitised paper can be regulated at will, and is capable of being varied from 6 inches per second to $\frac{1}{16}$ inch per second.

It will be noted from fig. 1, that the diaphragm which receives the body impulses is very highly damped by the rubber ring that supports it. The microphone container is purposely made double, with rubber connection, since the latter will damp out very efficiently any resonance the air in the system might have.

The vibration galvanometer is heavily shunted so that, although sensitivity is sacrificed, the instrument is practically dead-beat. Fig. 2 shows the record of a make and break of 1 ohm change in the resistance of the microphone. This record also indicates sensitivity, and enables us to standardise all records taken from day to day (Plate 5).

With a standard microphone grid, working with a given electric current, and with a galvanometer of one definite sensitivity, quantitative comparisons of the various records can be made. The recording of minute air currents by the microphone is a special feature of our method as distinct from that employed by Yandell Henderson, and has been dealt with in a paper by one of us (W. S. T.) and Capt. E. T. Paris, on "The Selective Hot-Wire Microphone" (15).

The air, with its small inertia, here takes the place of the mechanism of the swinging table. The movements of the air are recorded faithfully and without measurable lag by the change in the temperature and electrical resistance of the hot-wire microphone.

The microphone is subject to resistance variation through change in the temperature of the room, thus changing the zero. This effect, however, can be overcome by simply adjusting the balance of the Wheatstone bridge.

In order to use the microphone—which has a resistance of about 150 ohm when heated—it is inserted in one arm of a Wheatstone bridge, the other arm being adjusted by a rheostat to give balance with the string galvanometer. The pulsating currents of air which cool the microphone grid, give a corresponding variation of resistance, but it must be noted in this investigation that motions of the air, whether positive or negative in direction, always produce positive deflections on the galvanometer wire, since all air movements produce a fall in temperature and a diminution in resistance of the microphone grid.

Another point to be emphasised is that deflections of the galvanometer wire are not proportional to the displacements of the body in a given direction, but are proportional to some function of the velocity of the body under recoil. What this function is, may be indicated by reference to the previous work done on the theory of the hot-wire microphone.

The deflection of the galvanometer, which may be considered to be proportional to the change in electrical resistance of the microphone, is dependent not only on the vibratory motions of the air set up by the heart action, but also on a certain amount of direct air current set up by convection, as a result of the disposition of the microphone grid in the orifice through which the vibrations are transmitted. Convection effect was reduced to a minimum in this investigation, by setting the plane of the microphone grid vertical, so that convection currents tend to be perpendicular to the displacements of the vibrating air. These currents, however, could not be completely eliminated, owing to the lack of complete symmetry of the enclosure on the opposite sides of the grid.

It has been shown in the paper above referred to that for a certain type of grid similar to that in use and under similar conditions, receiving vibrations $U \sin pt$, the total resistance change

$$\delta R = -0{\cdot}15\, U^2 + 0{\cdot}15\, U \sin pt + 0{\cdot}15\, U^2 \cos 2pt,$$

where U is the velocity of the air at any moment past the grid, $2\pi/p$ the period of vibration of the air. Other terms may be added to the above series, but have been shown to be so small as to be negligible. The convection current in direction parallel to the displacements of the vibrating particles, is entirely responsible for the term containing U sin *pt* and from the equation it is seen that a simple harmonic vibration in the air is recorded by :—

(i) a displacement of the mean position proportional to the square of the velocity;

(ii) a periodic term in tune with the air vibration, of amplitude proportional to the velocity;

(iii) a periodic term an octave above this vibration proportional to the square of the velocity.

Terms (i) and (iii), therefore, are proportional to the kinetic energy in the air, while (ii) is proportional to the square root of that energy.

This relation has been checked by means of an artificial vibrating system fixed to the platform referred to above, and consisting of a spiral spring supporting a weight which, when displaced, vibrates vertically up and down. The spring becomes, for the time, an artificial heart, imparting its vibrations through the platform to the diaphragm and to the air in the microphone chamber.

The diaphragm is put under the same tension, etc., by placing weights upon the platform equal to the weight of the case under comparison.

Fig. 3A gives the galvanometer record which closely agrees with the above equation in which the maximum velocity of the vibrating air is 2 cm. per second, and the convection current about 2 cm. per second (Plate 5).

One complete vibration corresponds to two peaks, the higher one of which indicates a displacement of the vibrating air in the same direction as the air current.

At any point, the deflection of the galvanometer is proportional to the function of U as quoted in the above equation, but if the function be integrated with respect to time for a complete cycle, *i.e.*, for a time $2\pi/p$ the periodic terms vanish and a quantity proportional to U^2, *i.e.*, to the kinetic energy of vibration, is obtained.

If now the amount of kinetic energy contained in the spring is calculated from the mass of the spring, its periodic time and its displacements, we can calibrate the records in terms of such kinetic energy.

The vibrating spring, however, fails to resemble the heart in its action since, as seen from the record, the spring loses very little energy per period, *i.e.*, it is a nearly undamped vibrating system. Had it been heavily damped, the spring would rapidly come to rest and the record would show markedly decreasing amplitudes.

With the heart quite a different condition obtains. The projected blood constitutes the mass of the spring and the muscles of the heart the spring itself, but the energy which is imparted to the blood, and therefore to the body as a whole, is immediately absorbed so that the vibrations appear to be dead beat. It is nevertheless true that the resistance variation, though not to be expressed by so simple an equation as that quoted above, can be treated in a similar manner, and for a heart cycle, consisting as it does of a number of vibrations, superimposed or consecutive, between two so-called heart beats, a process of integration can be carried out, and the result will give a quantity from which the periodic terms vanish, and which has a value proportional to a $(\text{velocity})^2$, *i.e.*, to the kinetic energy imparted to the body during the cycle.

The process of obtaining the kinetic energy of recoil of the body resolves itself, therefore, into the measurement of an area bounded by the heart curve and the zero axis, dividing this by the time, and expressing the result in any desired units of energy, by comparing with a similar area divided by time, given by the vibrating spring of known energy.

The kinetic energy contained in the spring was found from the relation $\frac{1}{2}\mathrm{M}p^2d^2$, where d is the amplitude of the spring and M the effective mass which is maintained in vibration with a periodicity of $p'/2\pi$. This corresponds during calibration to an amplitude in the Einthoven string of a.

The photographic record shows an amplitude of b, so that the kinetic energy of the spring as recorded is:—

$$\mathrm{M}p^2d^2b/2a.$$

In the case under consideration this quantity works out to 22×10^4 ergs and may be measured by the mean ordinate of the spring curve—say Y.

Dealing now with the heart curve, examination is made over a complete breathing cycle of six heart beats, and the mean ordinate for these is obtained (fig. 3, B). Calling this y, the kinetic energy exhibited in the body corresponds to

$$y/\mathrm{Y} \times 22 \times 10^4 \text{ ergs}$$

and this is completely absorbed, the time corresponding to it being t seconds.

Hence the kinetic energy produced averages for a breathing cycle

$$y/\mathrm{Y}t \times 22 \times 10^4 \text{ ergs per sec.}$$

In this determination $y/\mathrm{Y} = \frac{1}{2}$ and $t = 4{\cdot}23$ secs., so that the heart output creates a kinetic energy in the body of

$$2{\cdot}6 \times 10^4 \text{ ergs per sec.}$$

or

$$0{\cdot}18 \text{ gramme-metres per heart cycle.}$$

The figures obtained do not represent the total kinetic energy of the heart but are, we suggest, proportional to it. They must not, therefore, be compared directly with the work done by the heart, which, as given by Starling (12), is in the neighbourhood of 82·3 gramme-metres per beat, of which 81·6 units represent work done against arterial pressure, and 0·7 units are measured in kinetic energy at the root of the aorta. The latter again is considerably greater than the value obtained by our experiments, since our measurements are integral of all movements footward and headward.

One other point should be referred to before proceeding. Yandell Henderson obtained records of displacement variation, and these in a given mass, that of the patient with the platform that supports him, are also records of the potential energy of that mass.

Thus it will be seen that the measurements made by Yandell Henderson and ourselves are complementary, since in any vibrating system the total energy at any time is the sum of its potential and kinetic energies. The two quantities will be out of phase $\pi/2$, maximum potential energy corresponding to minimum kinetic energy, and *vice versâ*.

The peaks of the displacement curve correspond to the minima of the records of this paper. Also, while the displacement curves can indicate positive and negative values, the curves of our records only give positive values. Allowing now for the change of phase of $\pi/2$ on passing from one curve to the other, and remembering that the values of the kinetic energy curves are always positive, it is possible to show a resemblance between the two sets of curves.

Fig. 4 is a reproduction of a diagram from Yandell Henderson's paper. Taking fig. 3, B, to represent a characteristic heart record obtained by the process described in this paper, the curve of fig. 4 can be approximately reproduced by reversing alternate peaks to allow for positive and negative

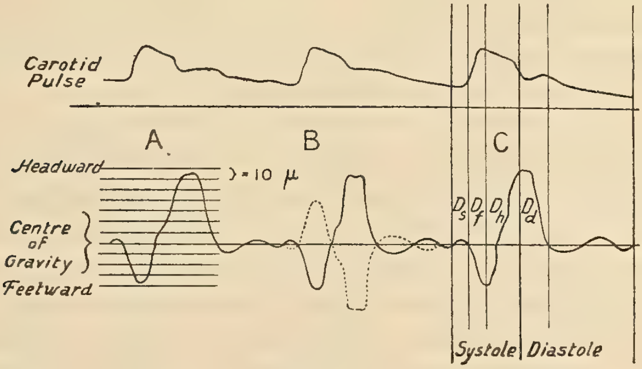


FIG. 4.

effects. The change of phase would be imposed by moving the curves as a whole through the width of half a peak, bearing in mind also that whereas

Yandell Henderson's curve reads from left to right, the record of fig. 3 reads from right to left.

Theoretical Considerations.

The maintenance of life depends ultimately upon the efficiency of the circulation.

Since circulation is maintained by the pumping action of the heart, any measurement of its output gives an effective method of testing the controlling engine of the body.

Yandell Henderson used the displacements in the body recoil in an attempt to measure the volume discharged from the heart per unit weight of the body at each contraction. Thus, a body of weight W would suffer a displacement D, corresponding to the propulsion of blood of weight w displacing an amount d at each heart contraction, where

$$w = \mathrm{WD}/d.$$

With accurate measurements of body displacement a figure is thus obtained proportional to the volume of the discharge per systole. It is obvious, however, that the actual work done in producing the movements of the body is not the reaction movement of the left ventricle only, but the algebraic sums of all the movements of blood or body fluids during the period of estimation.

The difference between our measurements and those of Yandell Henderson can now be clearly indicated. His curves are effective indications of the potential energy in the body at any moment, and changes in such energy are proportional to systolic discharge. The curves we obtain are indications of the complementary kinetic energy of the body, changes in which are proportional to the changes in potential energy. If Yandell Henderson's curves, therefore, measure systolic discharge, the same claim can be made for the curves given by the hot-wire microphone.

The advantage of obtaining kinetic energy curves rather than those of displacement shows itself in one important way. The displacements of the body produced by causes other than those due to heart action may be considerable. In the act of breathing, for example, the movements obtained may be many times greater than those due to propulsion of the blood. For this reason, Yandell Henderson took special precautions, such as holding the breath or causing the patient to blow steadily through a whistle. In our case, however, we are concerned with velocities rather than displacements, and the velocities in the body resulting from breathing are so small as to be negligible. The same applies also to slow muscular movements in the body, incidental on digestion, etc. Velocities of the air resulting from the diaphragm displacement in the microphone container are here exceedingly minute, and we are thus able to assume that they produce no appreciable change in the electrical resistance of the microphone.

We are able to show, however, that breathing does produce a marked effect in systolic discharge, as will be clearly indicated in the records described later.

Experimental Work.

The earliest records were obtained by the simple process of seating the patient on a microphone container. It was found that the "spring" of the walls sufficed to produce measurable effects. In order to avoid change in zero of a balanced electric circuit, the resistance variations were conveyed to the galvanometer through the agency of a transformer.

The next development was that in which the patient was seated in a chair which was bolted to a wall of the container at a point beneath its centre of gravity. Both of these methods involved careful balance of the body, and in any case could give no quantitative results. The chair was then slung by chains from the under surface of the diaphragm in the apparatus described earlier in the paper, and a foot rest was fitted so that the patient could be seated at ease. Here, again, the effect, though more consistent, and though to some extent capable of comparable measurement, depended on the attitude of the subject, and records showed that marked differences were given when the knees were bent to different extents, or when the head was erect or bowed. Finally, the subject to be tested was placed on the swinging platform, in an absolutely erect position, with a shoulder rest to steady the body.

A change was also made in the electrical arrangements. It was found possible to replace the transformer method by that of the simple Wheatstone bridge. This latter arrangement enabled us to get more faithful records of the resistance changes of the microphone. Contrary to expectation, the zero of the record was found to be quite steady.

Consideration and Analysis of the Curves.

The Normal Recoil Curve.—The normal recoil curve is undoubtedly very similar to that shown in fig. 5. This curve and the majority of those reproduced in this paper have all been obtained from one subject (Mr. A. Reading), whose general physical standards are equal to those found in the best type of pilot. In addition, the electro-cardiogram and orthodiagrams of this case are normal (Plate 5).

Very similar records, which need not be reproduced here, were given by many other normal subjects.

As will be seen from the figure, the main features of the normal curve consist of (reading from right to left) a small peak, followed immediately by two considerably larger peaks, and then by two smaller and somewhat flatter peaks. These peaks have been numbered 1 to 5 in the diagram. These five peaks, since they are nearly always present in every curve, may be considered as the essential elements of the normal recoil curve. In some of the heart cycles in the figure other smaller peaks may be observed, and it is generally these secondary peaks that tend to become exaggerated, or entirely absent, from case to case.

Fig. 5 shows also a simultaneous record taken over the carotid by means of the special hot-wire microphone previously described. On this record, the time relations of the various peaks of the recoil curve in relation to the phenomena of a heart cycle have been purposely omitted. This has been done to avoid confusion and error, as the peak of the "hot-wire" carotid curve, which indicates the maximum of velocity, does not occur at the same time as that on the ordinary carotid curve. It is, as shown previously, a curve out of phase with the ordinary curves which record changes in pressure. When the simultaneous records of figs. 5 and 6 were taken we had no comparator at our disposal and therefore have not given an exact figure for the time interval between the commencement of the first heart sound and the commencement of the carotid peak.

In the heart-sound microphone, the deflections caused by the commencement of the first and second sounds, corresponding, as they do, to the closure of the auriculo-ventricular and semilunar valves, fix very definite points of time in the heart cycle for comparative purposes on simultaneous tracings.

As heart-sound records are free from the difficulty inherent in any comparison between potential and kinetic energy curves, a simultaneous record of recoil and heart sounds has been prepared, and this is shown in fig. 6, while the relation of the two sounds has been more clearly defined by lines drawn through their commencement to cut the recoil curve.

As it is undesirable to introduce the recoil curve into the standard composite diagram representing changes of pressure in auricles, ventricles, aorta and carotid, a special diagram, fig. 7, has been prepared, in which the kinetic energy curves of body recoil and carotid are co-ordinated with heart sounds. For the time relations of the various events in a heart cycle, the data given by Lewis (10) have been used.

From figs. 5 and 7 the commencement of the carotid curve is seen to coincide with the beginning of the first or small peak of the recoil curve. This peak (1) is, therefore, probably caused by the feetward movement of the body corresponding to the headward movement of blood as it is projected from the left ventricle shortly after the beginning of each systole. The interpretation of the two peaks (2 and 3) is, we think, as follows: The first is probably due to the movement of the body headwards as the mass of blood passes down the aorta, while the second is caused by the return of the displaced body to normal.

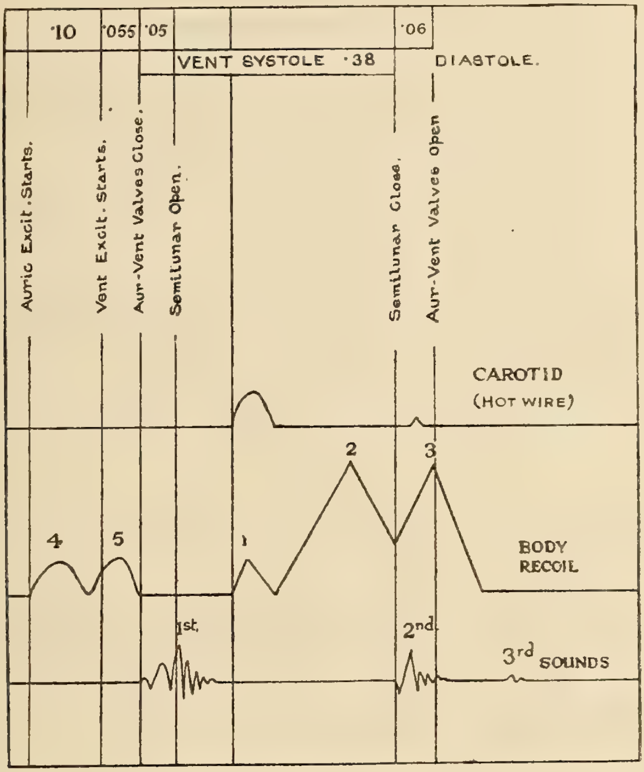

·10
·055
·05
·06
VENT SYSTOLE ·38
DIASTOLE.
Auric Excit. Starts.
Vent Excit. Starts.
Aur-Vent Valves Close.
Semilunar Open.
Semilunar Close.
Aur-Vent Valves Open
CAROTID
(HOT WIRE)
2
3
4
5
1
BODY
RECOIL
1st.
2nd
3rd SOUNDS

FIG. 7. (Read left to right)

The interpretation of the two smaller peaks (4 and 5) is, however, not easy, as it is difficult to believe that a recoil of this magnitude can be produced entirely by auricular systole.

In this connection, it should not be forgotten that patients with large and heavy hearts, not necessarily dilated *post-mortem*, frequently shake the whole bed. The question, therefore, arises whether, or not, some of the features of the recoil curve are due to the actual movement of the heart itself. It has been shown by needles passed into the base centre and apex that the base moves downward during systole. The elastic recoil of the distended aorta also requires taking into consideration. These questions have been left for subsequent investigation.

The interpretation put forward here of the recoil peaks must be considered. at present, as purely tentative.

In fig. 5 it will be seen that the recoils increase and decrease at regular intervals of time, and the relation of these increases and decreases to respiration are well shown in fig. 8. In this figure, the shorter parts of the breathing record correspond to inspiration, and it will be seen that, in agreement with other physiological observations, the maximum recoil occurs just after expiration has commenced (Plate 6).

The effect on the recoil curve, when the breath is held in deep inspiration and deep expiration, is well shown in fig. 9.

The recoils, which are so largely increased in inspiration and diminished in expiration, are undoubtedly due to the normal physiological variations in output during respiration. During inspiration, the descent of the diaphragm increases the positive pressure in the abdomen, thereby tending to press blood out of the abdominal veins, and at the same time the negative pressure in the thorax is increased, and greater suction exerted. The heart, therefore, will be better supplied during inspiration with blood than during expiration, and in consequence the output will increase, and enlarged recoils be the result.

The variation in curves given by apparently healthy individuals may be seen from fig. 10, where the first example demonstrates a simplified type of curve, in which only the primary peaks are recognisable, and from which secondary peaks are practically absent (Plate 7).

The second example shows the type in which the secondary peaks are so enlarged that they occasionally make recognition of the primary peaks difficult without dividers.

The third example, taken very shortly after the two upper curves, and without any alteration of the apparatus, is from the standard normal subject (A. Reading).

The meaning of these variations is the subject of investigation along two lines, one anatomical, and the other physiological. Small variations in the anatomical position of the axis of the heart, or in the disposition of the great vessels, may, as referred to above, alter the proportion of the total kinetic energy of a contraction acting in the long axis of the body. Or, as Krogh and Lindhard (9) have shown, individuals may have a high or low coefficient of oxygen utilisation, with a consequent small or big volume output from the heart, *i.e.*, a small or big recoil curve.

Variations in Blood Pressure.—As soon as it became evident that the recoil curves were subject to wide normal variations from subject to subject, a certain number of experiments were carried out to determine the variations which made their appearance in the normal subject under different conditions.

Curves were therefore taken before and after meals over a series of consecutive days at the same hour; and before and after exercise.

It was thought at first that the variations in the recoil might bear some simple relation to the blood pressure. A number of healthy individuals were, therefore, examined from this point of view, but yielded no definite evidence that small differences of systolic, diastolic or pulse pressure readings could be correlated with alterations in the size of the recoil.

As Tigerstedt (13) had shown, with his *Stromuhr*, that nitro-glycerine increased the minute-volume and lowered the blood pressure, while adrenalin tended to the opposite effect, it was decided to try the effect of small doses of vaso-constrictors and vaso-dilators, liquor adrenalin and nitro-glycerine respectively, taken by the mouth. Accepting Tigerstedt's results as applicable to the normal human subject, it would seem that enlarged recoils are the result of increases in volume output rather than raised blood pressure, as is clearly shown by fig. 11. In this figure, the only curve showing a definite increase of recoil is (*d*), *i.e.*, the one taken five minutes after a small dose of nitro-glycerine. Curve (*c*), taken after a small dose of adrenalin, is not appreciably different from any of the control curves.

It is true that the dose of liquor adrenalin is very small, and was given by the mouth, but the effect of the nitro-glycerine is very striking.

Curves were also obtained after taking by the mouth 0·02 grm. of the vaso-constrictor tyramine, a drug which is definitely known to be absorbed, and recoils of diminished amplitude were obtained.

The results are not conclusive, but, taken in conjunction with the variations during respiration, are suggestive that alterations in the volume output are the chief factors affecting the magnitude of the recoil; this agrees with the theory of the action of the microphone dealt with above.

The result of experiments carried out to show the effect of increased heart work on the recoil indicates that all the features of the normal curve become enlarged, especially the smaller peaks. An example of a curve taken before, immediately after, and some time after exercise, is shown here to illustrate this point (fig. 12, Plate 8).

It would not, however, be justifiable to assume, at this stage, that a curve such as (B) of fig. 10 is produced because the subject's heart is doing more work.

All the experiments that have been carried out have been directed solely towards ascertaining the nature of a normal curve, and the variations to which it is subject. As a single control to these experiments, the recoil curve from a case of aortic regurgitation with a high blood pressure, and a very large heart, was taken. It is shown here (fig. 13) as an example of the extreme deflection caused by the hot-wire microphone, when abnormal movements of the body are transmitted to the air in contact with it.

Conclusion.

A careful examination of the above records shows that there is an additional periodic effect, besides that of the heart cycle and of breathing. This reveals itself in the variation in heights of the two highest peaks. At certain parts of the record, the first peak is higher, in other parts it is lower than the second peak, while we also get the intermediate conditions of equality of peaks. One of these effects can be partially explained by the difference in sensitivity of the microphone according to the direction of the air current; but some physiological explanation is required to account for the whole phenomenon, as instrumental variations have been reduced to a minimum. This effect will be the subject of further study.

The apparatus employed for the recoil measurements is now being further modified, so that the patient can be examined in a recumbent position. Records could then be obtained when the muscles are completely relaxed and might easily be taken during sleep.

This further modification will also permit us to measure the recoil in directions perpendicular to the body axis, so that information regarding the whole of the body movements should then be available.

Summary.

This paper deals with a new method of measuring body recoil as the result of heart action. Attempts have been made to eliminate the disturbing factors operating against the success of Yandell Henderson's method. To effect this, the hot-wire microphone with suitable galvanometer and recording apparatus has been employed, and the records actually made measure quantities proportional to the kinetic energy imparted to the body by motions of the blood. In this way slow-moving displacements, such as those of breathing, fail to be recorded.

The apparatus is of such form that it can be standardised, giving the same responses from day to day for the same body recoils.

A method is indicated of expressing this kinetic energy of the body in C.G.S. units. Attention has been concentrated on the records obtained from a favourable subject, and an analysis of these curves shows that the events of a heart cycle can be recognised.

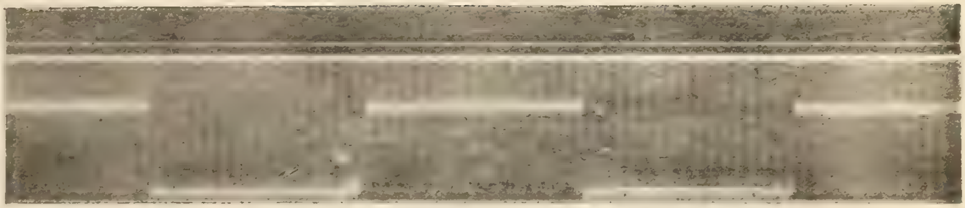


Fig. 2

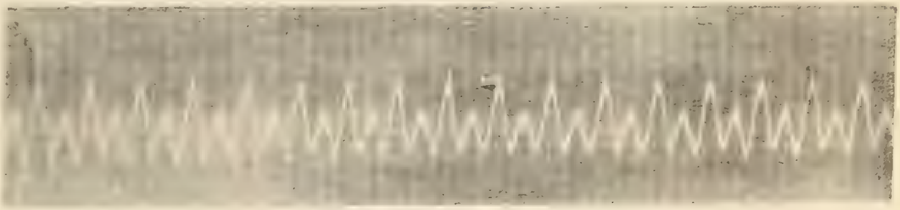


Fig. 3 A

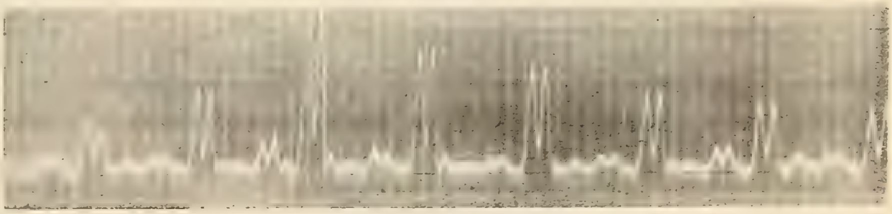


Fig. 3 B

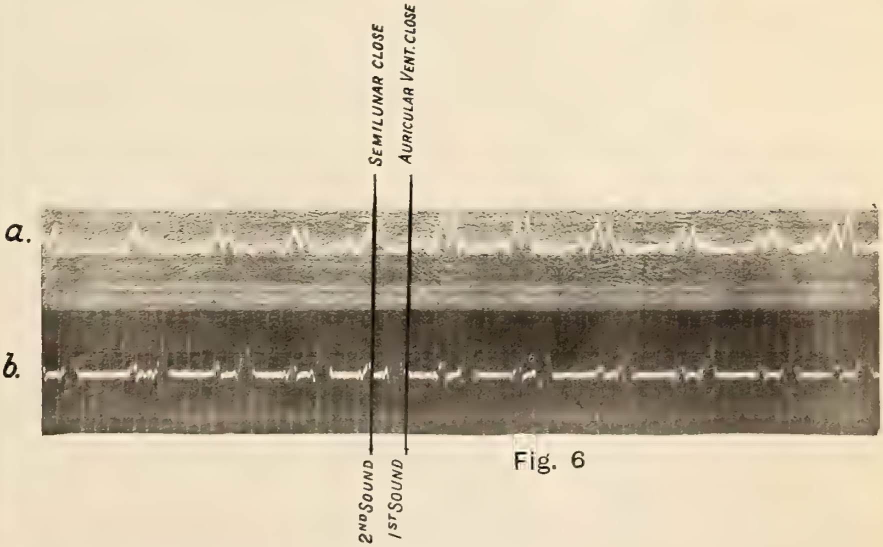


Fig. 6

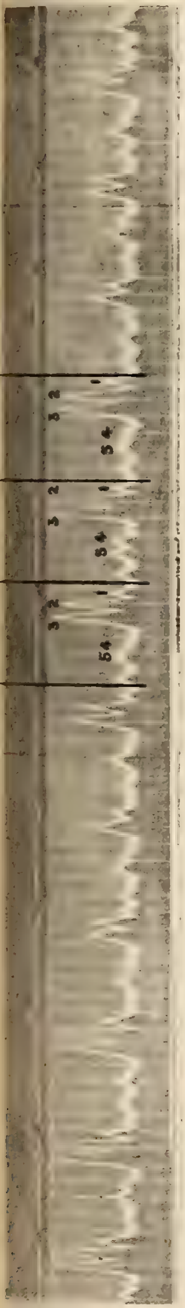


Fig. 5

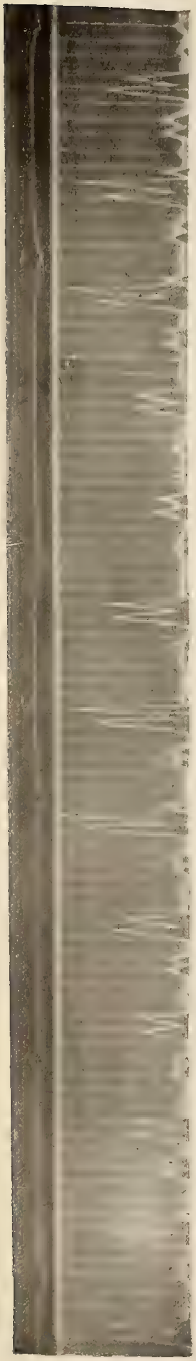


Fig. 8

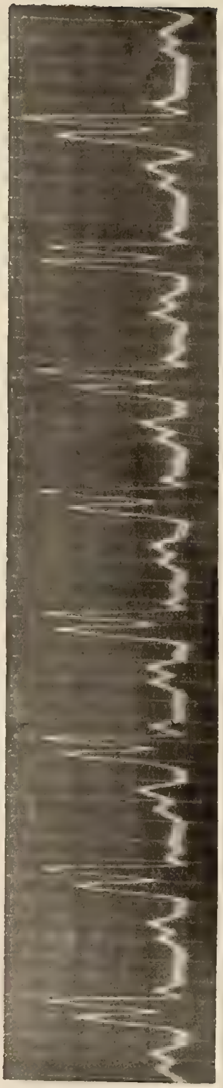


Fig. 9 INSPIRATION (held)

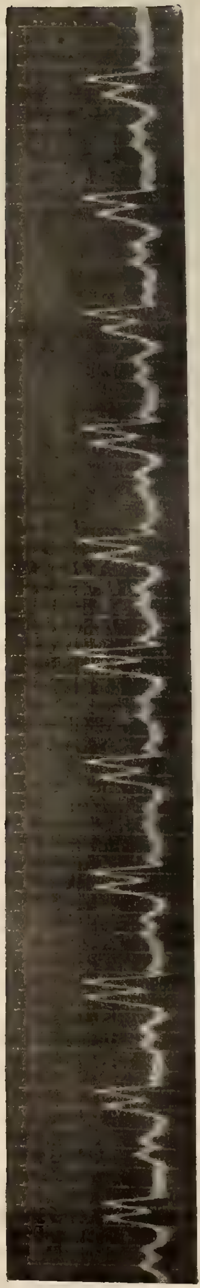


Fig. 9 EXPIRATION (held)

Fig. 10

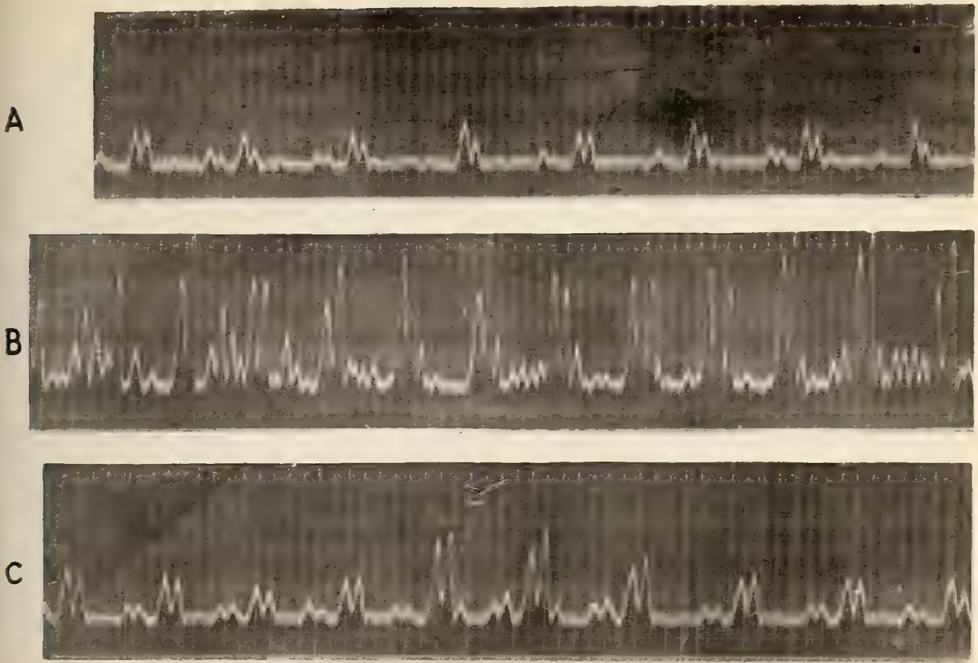
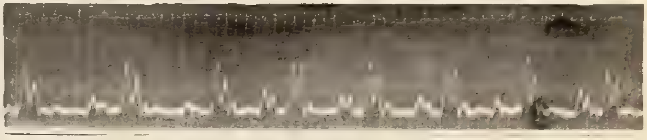
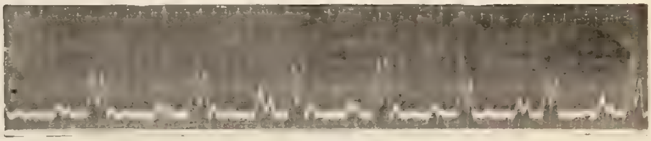
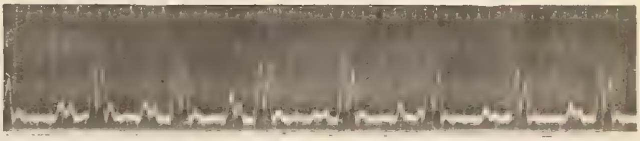
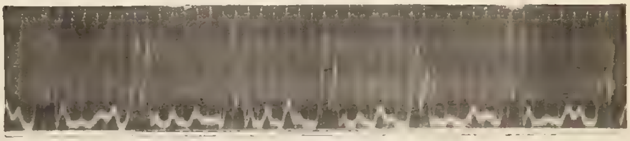


Fig. 11


(a) Normal Recoil Curve as general Control.


(b) 5 minutes after 1 ounce of Peppermint Water (to demonstrate presence of a psychological effect, if any). Showing no psychological effect produced.


c) 5 minutes after 1 ounce of Peppermint Water, to which 15 drops of Liquor Adrenalin had been added.


(d) 5 minutes after 1 ounce of Peppermint Water, to which 1/150 grain of Nitroglycerin had been added. Showing great increase in second and third peaks.

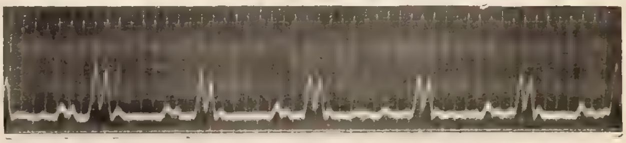
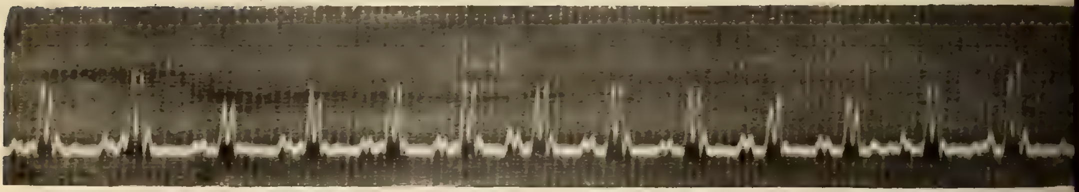
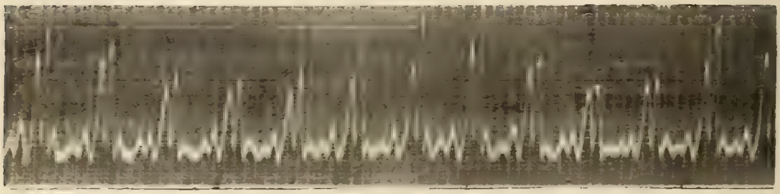

(e) Control curve taken half-an-hour after (d), showing complete return to normal.

Fig. 12

NORMAL HEART RECORD BEFORE EXERCISE



IMMEDIATELY AFTER EXERCISE



60 SECONDS AFTER EXERCISE

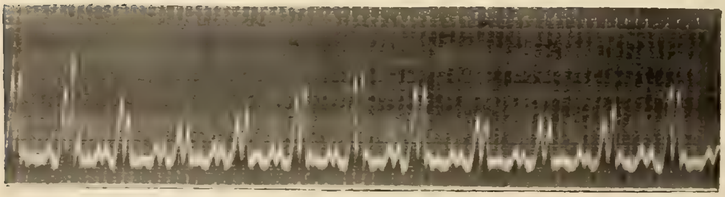
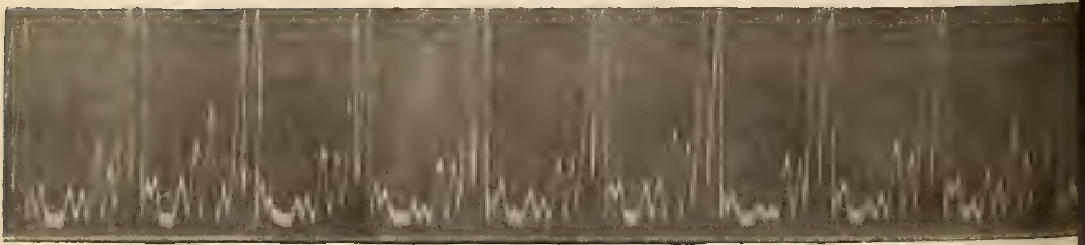


Fig. 13



The results so far obtained are consistent with accepted physiological data as to the variations in the systolic output of the heart, as affected by exercise, respiration or the action of vaso-constrictors and vaso-dilators.

In conclusion, the authors wish to express their appreciation of the help and assistance given to them in carrying out this work by Sir Frederick Sykes, the Controller-General of Civil Aviation, and Lieut.-Colonel Cusins, Chief Experimental Officer of the Signals Experimental Establishment, where the work was carried out. They further desire to express their indebtedness to Prof. Yandell Henderson, Dr. A. W. Stott for his kind collaboration during the preliminary stages, and to Dr. P. Hamill, when correlation with the electro-cardiograph was required, as well as for advice on the physiological aspects of the paper.

The authors particularly wish to recognise the help given by Mr. A. Reading, who, besides providing them with a very useful subject for heart recording, also manipulated the apparatus most skilfully.

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The Velocity of the Pulse Wave in Man.

By J. CRIGHTON BRAMWELL,* M.B., M.R.C.P., and A. V. HILL, F.R.S.

(Received February 4, 1922.)

In an investigation now being carried out by us at Manchester observations are being made, under various conditions, upon the velocity of the pulse wave in man. As a preliminary to this investigation it was thought advisable to study the theory of the transmission of the pulse wave, and the following pages contain the results arrived at, together with an account of experiments upon the velocity of the pulse wave in an isolated human artery.

The pulse wave in man travels in the arteries at a speed of 4 to 10 metres per second. Its velocity depends, to a small degree, on the velocity of the blood in the artery considered, but chiefly upon the elastic condition of the arterial wall, which is affected by a variety of factors in health and disease. As regards the former, the pulse wave must be considered as travelling, like a ripple on moving water, relatively to the fluid in which it occurs. The arterial wall merely exerts an elastic constraint upon the surface of the fluid, and in the simplified theory of the transmission of the wave (which it is necessary for practical purposes to adopt) the inertia of the wall, and of the tissues outside it, exerts no influence on the velocity of the wave. Thus any experimentally determined value must represent the velocity of the wave relatively to the blood, *plus* the velocity of the blood in the artery. Taking 0·75 metres per second as an average maximum velocity of the blood in the aorta, and 0·25 metres per second as an average maximum in the carotid artery (4), we see that the correction for the velocity of the blood itself is small, but not negligible, in comparison with the velocity of the wave. Any considerable increase in the velocity of the blood, caused, *e.g.*, by local or general exertion, will cause an equal increase in the velocity of the pulse wave.

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* Working for the Medical Research Council.

Moreover, the velocity of the blood in the aorta, and to some degree in any artery, varies considerably at different moments of the cardiac cycle; such differences will cause one part of the pulse wave to be transmitted with a greater velocity than another, and so will lead to a certain modification in the apparent form of the wave. In the absence of definite knowledge of the velocity of the blood in any given case it is not possible to make any allowance for it; it is necessary, however, to bear in mind that it may, under certain circumstances, appreciably—though not considerably—affect the velocity and modify the form of the transmitted wave.

Considered in its full complexity the theory of the transmission of the pulse wave is difficult. There are, however, two factors which allow us to simplify it: (*a*) the distance over which the wave travels is relatively short; (*b*) the wave form, owing to the elastic nature of all the tissues producing it, shows no very sharp discontinuities or changes of curvature. In consequence of (*b*), in the analysis of the wave into a system of simple harmonic waves, the shorter wave-lengths are relatively unimportant, and it is the transmission of these waves which would have required the more complicated treatment. With the help of Mr. E. A. Milne, of Trinity College, Cambridge, a fuller theory of the wave transmission has been worked out; it is unnecessary to give this theory at length, but it may be stated that, with the type of wave occurring in arteries, and within the limits of experimental error, the formula given by Moens (7) in 1878 is sufficiently accurate for our purpose. We will consider the meaning and application of this formula.

If v be the velocity of the front of the pulse wave, y the radius of the artery at the end of diastole, c the thickness of the arterial wall, E the modulus of elasticity of the artery for lateral expansion, and ρ the density of the blood, the following relation holds:

$$v = \sqrt{(\mathrm{E}c/2\rho y)}.$$

Assuming that ρ is constant, and equal (say) to 1·055, this formula contains three variable factors, on which the value of v depends, viz., E, c and y. In this form the expression is of little value, since E, c and y vary from artery to artery, and none of them express any easily measurable factor. By a simple transformation, however, a formula may be obtained which throws much light upon the mechanics of the circulation. A small rise δp in pressure may be shown to cause a small increase, $\delta y = y^2\delta p/\mathrm{E}c$, in the radius y of the artery, or a small increase, $\delta \mathrm{V} = 2\pi y^3 \delta p/\mathrm{E}c$, in its volume V per unit length. Hence $2y/\mathrm{E}c = d\mathrm{V}/\mathrm{V}dp$, from which

$$v = \sqrt{(\mathrm{V}/[\rho d\mathrm{V}/dp])}.$$

In this equation p is measured in dynes per square centimetre, and v in centimetres per second. Expressing p in millimetres of Hg, and v in metres per second, and substituting $\rho = 1{\cdot}055$, this equation becomes,

$$v = 0{\cdot}357\sqrt{(\mathrm{V}/[d\mathrm{V}/dp])}.$$

But $(d\mathrm{V}/dp)/\mathrm{V}$ is the relative increase in the volume of the artery, per millimetre of Hg increase of pressure. Working in percentages, therefore, the equation finally becomes

$$v = 3{\cdot}57/\sqrt{(\text{percentage increase in volume per millimetre of Hg increase of pressure})}.$$

This is the form most intelligible and convenient in use. It requires no knowledge of the elastic coefficient as such, nor of the radius and the thickness of the arterial wall, but only of one simple and directly observable function of these, the rate of increase of volume with pressure. Thus an observation of the velocity of the pulse wave in any particular vessel tells us at once, in absolute units, the degree of extensibility of that vessel.

The energy expended by the heart, per beat, has been shown by Rhode (2) and others, to depend (other things being equal) on the pressure developed by it. Thus, if the heart is to work efficiently, the output for a given pressure should be as large as possible, which implies a large increase in the volume of the arteries per millimetre of pressure developed, and—from the formula—a low velocity of the pulse wave. Another sign of an efficient circulation is that the flow through the capillaries should remain as high and as constant as possible during diastole, which implies a large diminution of volume of the arteries for a given fall of pressure, and again a low velocity of the pulse wave. Hence a low velocity of the pulse wave is a sign, both of an efficient and continuous circulation and of an economical functioning of the heart. Thus the velocity of the pulse wave is one important criterion of the general efficiency of the circulation.

In a paper by Roy (3), in 1880, is given a series of curves showing the relation between volume and pressure, in the case of arteries and veins, made by an ingenious method, commanding every confidence in its accuracy. Replotting these curves in rectangular co-ordinates, and measuring their slopes at various points, it is possible to deduce the percentage increase in volume per millimetre of Hg, and so to calculate the velocity of the pulse wave at various pressures. The following results are obtained by so doing :—

Table I.

I. Fig. 6, Roy's Paper.—Inferior Vena Cava of Cat.

p (mm. of Hg)	5	10	20	30	40
v (m.p.s.)	1 ·55	2 ·2	3 ·1	4 ·3	5 ·0

II. Fig. 7.—Femoral Artery of Rabbit.

p	20	40	60	80	100	120	140	160
v	2 ·21	2 ·27	2 ·12	2 ·33	3 ·10	5 ·05	7 ·15	9 ·22

III. Fig. 9.—Carotid of Rabbit, immediately after death.

p	20	40	60	70	80	100	120	140	160
v	3 ·15	3 ·15	3 ·08	3 ·1	3 ·48	4 ·7	7 ·0	10 ·7	17 ·8

IV. Fig. 11.—Thoracic Aorta of Cat.

p	20	40	60	80	100	120	140	160
v	3 ·4	3 ·6	3 ·5	3 ·6	3 ·6	3 ·8	4 ·3	5 ·4

V. Fig. 10.—Carotid of Emaciated Dog, suffering from ill-treatment and chronic illness.

p	20	40	60	80	100	120	140
v	3 ·1	4 ·0	5 ·1	6 ·0	7 ·3	8 ·4	8 ·9

The most striking fact about these figures is that in a normal healthy artery (II, III, and IV) the velocity is constant as the pressure rises from a low value up to about 80 mm., after which it increases, at first slowly and then more rapidly. At high pressures the velocity is very considerably increased. In V the velocity increases considerably throughout. Secondly, the velocities in II, III, and IV, at pressures of 80 mm. (about equal to the normal diastolic pressure in man), are noticeably less than those observed in man. This may be characteristic of the animals investigated, but it seems more probable that it is due to the following factor. All living tissues, and especially arteries and muscles, show the phenomenon of elastic "after-action," continuing to extend for some time if the load or tension be maintained. Roy attempted to avoid errors due to this by making his observations very slowly, allowing the tissue a long time to reach its final equilibrium. From the point of view of the static effect of the diastolic pressure on the arteries, he succeeded; from that, however, of the dynamic effects occurring in the rapid cycle of events associated with the pulse, his precautions aggravated the error, and must have caused the increase of volume per millimetre of Hg to be much larger than that occurring in a rapid change of pressure. It is quite conceivable that a pressure, lasting (say) for 0·1 second, causes an expansion not greater than half of that resulting from an equal pressure maintained for 10 minutes; in this case a calculated velocity based on the latter would be only about two-thirds of an observed velocity depending on the former. This elastic "after-action" therefore probably causes all the velocities in Table I to be too low; the effect is similar in character to that caused by adopting the formulæ for the *isothermal* expansion of a gas in calculating the velocity of sound. The safest thing to do is to measure the velocity directly, and so to deduce the constants of the true *adiabatic* expansion. Finally, we see (in I) that in a vein the calculated velocity at low pressures is very low, a conclusion which agrees with an observation of Morrow (6), and must be borne in mind in comparing the time relations of the jugular pulse with those of other events in the heart or circulation.

The most important point brought out by Table I is the dependence of the velocity upon the pressure. In the case of man, the pressure involved is the diastolic pressure, that on which the wave is superimposed. This implies a decrease in extensibility with increase in length, an effect analogous to that occurring in muscle. This is important in various ways, but particularly in experimental work, where it shows the necessity of recording the diastolic pressure at the same time as the velocity of the pulse wave. Its magnitude is emphasised in the experiment described below.

It is often suggested that in the living animal the velocity of the pulse wave may be affected by contraction of the involuntary muscle around the arteries. In so far as the contraction of involuntary muscle may affect the extensibility of the artery this will be the case, but in no other way. The part of the wave whose velocity is measured is the very rapid rise at the opening of the aortic valves, a rise which is detectible in a few thousandths of a second. It is inconceivable that a contraction of slow involuntary muscle, as we ordinarily know it, could affect the rate at which such a sudden rise is transmitted. The transmission of the pulse wave, therefore, is a purely mechanical phenomenon, its velocity being an indicator of the elasticity of the vessels, as modified by any conditions (muscular or otherwise), obtaining at the moment.

The chief difficulty in the observation of the velocity of the pulse wave in an isolated artery lies in the fact that no considerable length of artery can be obtained, and the time-interval available for measurement is therefore very small. By replacing the blood with mercury, however, this interval can be increased 3·58 times, and the utilisation of this fact makes it possible to measure the velocity in an isolated artery with fair accuracy, and then to obtain the velocity in an artery containing blood by multiplication by 3·58. The reason for this is as follows: the velocity of the pulse wave is inversely proportional to the square root of the density of the fluid in the vessel, so that replacing blood ($\rho = 1{\cdot}055$) with mercury ($\rho = 13{\cdot}5$) decreases the velocity in the ratio $\sqrt{13{\cdot}5/1{\cdot}055}$, *i.e.*, of 3·58:1. This principle is embodied in the

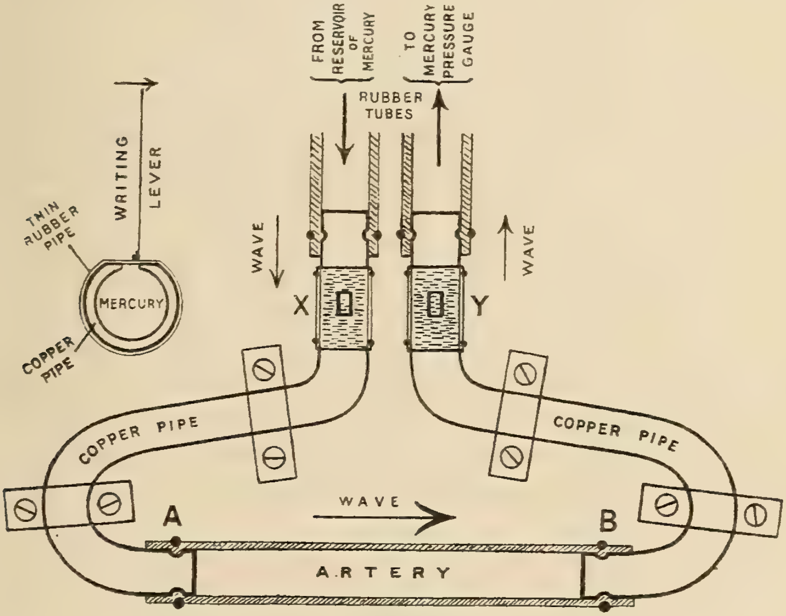

Fig. 1.

apparatus shown in fig. 1. A length of artery is tied firmly at A and B on to the copper pipes, which are clamped rigidly to a board. These copper pipes, at their other ends, are firmly joined to two long rubber tubes, one of which goes to a mercury reservoir capable of being raised and lowered, the other to a mercury pressure gauge. Thus the pressure in the artery filled with mercury (with bubbles carefully eliminated) can be adjusted to any required value. At X and Y in the copper pipes are two small windows, as shown in the diagram on the left. Over each window a small thin piece of rubber tube is carefully and firmly fixed, and on the rubber, over the *edge* of the window, is glued a minute aluminium angle-piece carrying the finest possible bamboo writing lever. The two levers, lying close together, write upon the same revolving drum, and indicate the moments of arrival of a wave in the mercury, at X and at Y respectively. The wave is set up by hitting or squeezing the left-hand rubber pipe at some distant spot. Its arrival at X causes a sharp movement of the writing-point at X, it is then transmitted with very high velocity through the almost rigid copper pipes, being delayed, however, in its arrival at Y by the slow transmission across the elastic artery between A and B.

After the experiment on the artery is completed, the artery is replaced by a rigid copper pipe from A to B, and the observations are repeated, the small time interval observed when the rigid tube lies on the path of the wave being subtracted from the larger interval when the elastic tube is there. In this way any small "zero error" in the instrument is practically eliminated. Corresponding points on the records of the two writing levers are compared, and the time interval between them can be determined with reasonable accuracy. Greater accuracy could doubtless be secured by employing a photographic method of recording the arrival of the wave at X and Y, but for the present purpose this was not necessary.

The artery employed was the common carotid of a young man (who had died of malignant endocarditis). It measured 6·84 cm. between the ends of the copper pipes. At each pressure a series of observations was made of the interval between the arrival of the wave at X and Y (fig. 1), and the "probable error" of the mean values given below, lay between 0·0005 and 0·001 secs. The "zero error" to be subtracted, as estimated by the interval observed when the artery was replaced by a copper pipe, was as follows:—

Pressure (mm.)	20	40	60	80	100	120	140	160	180	200
Zero error (secs.)	0·0135	0·0142	0·0150	0·0157	0·0165	0·0174	0·0182	0·0190	0·0198	0·0206

The following Table shows the results of a series of observations on the artery. The values for blood are obtained by multiplying by 3·58.

Pressure (mm.)	**25**	**57**	**78**	**92**	**110**	**152**	**200**
Interval observed (secs.)	0·079	0·086	0·0665	0·052	0·0465	0·0380	0·0338
Zero error (secs.)	0·014	0·015	0·0156	0·016	0·0170	0·0187	0·0206
Difference (secs.)	0·065	0·071	0·0509	0·036	0·0295	0·0193	0·0132
Velocity (metres p.s.)	1·05	0·96	1·34	1·90	2·32	3·55	5·18
Velocity, blood (m.p.s.)	**3·76**	**3·45**	**4·81**	**6·80**	**8·3**	**12·7**	**18·5**

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We see here the same effect of pressure as was shown by the calculation from Roy's curves: the velocity increases comparatively slowly as the pressure rises to about 80 mm., then more rapidly, and finally at high pressures very considerable velocities are observed. As regards the absolute value, we may compare the velocity given in the above Table with that found, at the same (diastolic) pressure, in a normal living subject. According to Gallavardin (5) the average normal value of the diastolic pressure in man is 70 to 75 mm. Much higher values, however, are given by the use of the Pachon oscillometer (80 to 110 mm.). In normal healthy young men our observations (to be described elsewhere) have given velocities from 5·8 to 7·4 metres per second. Compared with the velocity interpolated in the above Table, for a pressure of 70 to 75 mm., these velocities are high: accepting the higher estimate of the diastolic pressure in man, the observed velocities agree well with those given in the Table; the velocities 5·8 and 7·4 m.p.s. correspond roughly there to pressures of 85 and 102 mm., respectively. On the whole, therefore, we may be satisfied that the pulse-wave has a velocity in the living man not far different from that in an isolated artery, and that its transmission is a mechanical phenomenon depending only on the elastic properties of the vessels.

Summary.

The theory of the transmission of the pulse-wave in a blood vessel is considered, and it is shown that its velocity, in metres per second, is given by

$$v = 3{\cdot}57 / \sqrt{\text{(percentage increase in volume of artery per millimetre of Hg increase of pressure)}}.$$

This velocity is relative to the blood in the vessel, and must have a small correction applied for the velocity of the blood itself. An observation of the velocity, therefore, gives directly the degree of extensibility of the vessel, and is shown to be one criterion of an efficient circulation. The experiments of Roy (1880) on the extensibility of vessels may be used to calculate the velocity of the pulse-wave: the calculation shows: (*a*) that pressure has a considerable effect on the velocity, a fact which is confirmed by experiments on an isolated human artery, filled with mercury in order to slow the transmission of the wave; and (*b*) that the velocity so calculated is lower than observed in man, a fact which is attributed to the phenomenon of elastic "after-action," which affected Roy's measurements. The experiments on an isolated human artery gave a velocity comparable with that observed in man, and it is concluded that the transmission of the pulse-wave is a purely

mechanical effect, its velocity depending on the extensibility of the vessels as modified by any condition (muscular or otherwise) obtaining at the moment.

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On a Remarkable Bacteriolytic Element found in Tissues and Secretions.

By Alexander Fleming, M.B., F.R.C.S.

(Communicated by Sir Almroth Wright, F.R.S. Received February 13, 1922.)

(From the Laboratory of the Inoculation Department, St. Mary's Hospital.)

[Plate 9.]

In this communication I wish to draw attention to a substance present in the tissues and secretions of the body, which is capable of rapidly dissolving certain bacteria. As this substance has properties akin to those of ferments I have called it a "Lysozyme," and shall refer to it by this name throughout the communication.

The lysozyme was first noticed during some investigations made on a patient suffering from acute coryza. The nasal secretion of this patient was cultivated daily on blood agar plates, and for the first three days of the infection there was no growth, with the exception of an occasional staphylococcus colony. The culture made from the nasal mucus on the fourth day showed in 24 hours a large number of small colonies which, on examination, proved to be large gram-positive cocci arranged irregularly but with a tendency to diplococcal and tetrad formation. It is necessary to give here a very brief description of this microbe as with it most of the experiments described below were done, and it was with it that the phenomena to be described were best manifested. The microbe has not been exactly identified, but for purposes of this communication it may be alluded to as the *Micrococcus lysodeikticus.*

The fully developed colony of the coccus may be 2 or 3 mm. in diameter; it is round, opaque, raised, and has a bright lemon yellow colour; it grows luxuriantly on all the ordinary culture media, and growth takes place well at room temperature, or in the incubator at 37° C.; it is aerobic and facultatively anaerobic; it does not liquefy gelatin or coagulated albumin.

PRELIMINARY EXPERIMENTS SHOWING THE ACTION OF THE LYSOZYME.

In the first experiment nasal mucus from the patient, with coryza, was shaken up with five times its volume of normal salt solution, and the mixture was centrifuged. A drop of the clear supernatant fluid was placed on an agar plate, which had previously been thickly planted with *M. lysodeikticus*, and the plate was incubated at 37° C. for 24 hours, when it showed a copious growth of the coccus, except in the region where the nasal mucus had been placed. Here there was complete inhibition of growth, and this inhibition extended for a distance of about 1 cm. beyond the limits of the mucus.

This striking result led to further investigations, and it was noticed that one drop of the diluted nasal mucus added to 1 c.c. of a thick suspension of the cocci caused their complete disappearance in a few minutes at 37° C.

These two preliminary experiments clearly demonstrate the very powerful inhibitory and lytic action which the nasal mucus has upon the *M. lysodeikticus*. It will be shown later that this power is shared by most of the tissues and secretions of the human body, by the tissues of other animals, by vegetable tissues, and, to a very marked degree, by egg white.

FURTHER OBSERVATIONS ON THE EFFECT OF THE LYSOZYME ON BACTERIA.

1. *Inhibitory Action.*

In the preliminary experiments it has been shown that on the surface of an agar plate the growth of the nasal coccus is completely inhibited by superadded nasal mucus. This inhibitory action can be strikingly demonstrated in another manner.

A small portion of the agar is removed from an ordinary agar plate making a cup into which some material rich in lysozyme (tears, nasal mucus, sputum, cartilage, egg white, etc.) is placed. A drop of liquid agar, at a temperature of about 50° C., is placed on the material in the cup and is allowed to solidify, after which the cup is filled with the liquid agar which, in its turn, is allowed to set. Liquid agar is then poured all over the plate to make a thin layer over the original surface. The whole surface of the medium is now thickly planted with the *M. lysodeikticus* and the plate is incubated for 24 hours, when it will be seen that there is copious growth of the coccus, except in the region of the implanted material. By the method of preparation of the plate, in which the material is covered with several distinct layers of agar, there can be no mechanical transference of the material to the surface of the plate, but the experiment shows that the inhibitory substance is able to penetrate the agar and absolutely prevent growth of the coccus for a distance of about 1 cm. Further, if the plate is kept for a few days, it is found that portions of the growth next to the inhibition zone have become almost transparent, and it is evident that the lytic substance has continued to diffuse through the agar after the microbes have completed their growth, and has dissolved the cocci for a distance of 3 or 4 mm. The area of inhibition and the partially dissolved zone of growth are shown in Plate 9, fig. 1, which is a photograph of a plate in which was imbedded 10 c.mm. of tears.

2. *Bactericidal Action.*

If cultures are made from the inhibition zone of a plate, such as has been described in the last experiment, no growth results, showing that the bacteria implanted on this surface has been destroyed. It can also be shown that if lysozyme-containing material be added to a suspension of *M. lysodeikticus* in a test-tube these cocci are destroyed, so that cultures made from the tube remain sterile. In one experiment a suspension of *M. lysodeikticus*, of a strength of not less than 1,000 million per cubic centimetre, was exposed to the action of 1 in 100 nasal mucus and 10 c.mm. volumes were planted out after incubation for 1, 2, 5, 10, and 60 minutes. It was found that the cultures remained sterile while similar cultures made at the same time from a control tube in which the nasal mucus was replaced by normal salt solution, gave copious growth up to the end of the experiment, namely, 1 hour's incubation.

It was found that after 2 hours' incubation tears diluted 9,000 times with normal salt solution killed the whole of the cocci in a thick suspension of the *M. lysodeikticus*. In the dilutions of tears from 1 in 27,000 to 1 in 243,000 there was a very marked bactericidal power manifest.

The bactericidal action of the lysozyme may also be shown with microbes other than the nasal coccus. An example of this is illustrated in fig. 2, which is a photograph of a culture made after incubating a fæcal streptococcus for 2 hours at 45° C., with tears diluted in 1 in 100 and with normal saline solution. It will be seen that from the saline tube there resulted a continuous sheet of growth, whereas, from the tube containing the tears, there were only scattered colonies, showing that the vast majority of the streptococci had been destroyed by the tears.

A similar result was obtained by acting on *Streptococcus fæcalis* with the inflammatory exudation into a joint cavity.

Lytic Action.

Naked-eye Changes.—In the second of the preliminary experiments, it was shown that, if a drop of nasal mucus be added to a thick suspension of the *Micrococcus lysodeikticus* in a test-tube, there is, after a short period of incubation, a complete clearing of the opaque suspension, so that the fluid becomes perfectly clear to the naked eye. It has been noted also that other tissues and secretions have the same action. If the material used is rich in lysozyme, the action is a very rapid one. Thus, at a temperature of 45° C., a 1 in 100 dilution of tears will completely clear the suspension in about 30 seconds, or a 1 in 5 dilution of egg white in 10 seconds.

If the bacterial suspension is a very thick one, there is easily to be observed a considerable increase in the viscosity of the fluid after lysis of the bacteria has been completed, evidenced by the fact that, if the tube is shaken, the air bubbles rise much more slowly to the surface of the fluid.

The lytic action can be strikingly demonstrated by placing on the surface of a fully-grown plate culture of *M. lysodeikticus* a drop of tears, nasal mucus, or other material rich in lysozyme. In less than 1 minute at 37° C., or in about 10 minutes at room temperature, that portion of the culture on which the material was placed will have been completely dissolved, producing a clear space just as if a portion of the culture had been mechanically removed.

Microscopic Changes.—When a mixture of tears and *M. lysodeikticus* is observed with dark ground illumination, it is seen that the cocci rapidly lose their sharp outlines, become swollen and gradually disappear. At the same time, there appear a very large number of minute granules, somewhat similar in appearance to the granules of a polynuclear leucocyte.

Examined with transmitted light, it is seen that the cocci rapidly swell up and become transparent, so that, after 2 minutes at room temperature (when the cocci are suspended in undiluted tears), they become quite invisible.

When the partially dissolved cocci are examined by Burri's method, they are found to be much swollen up, and they are less indistinct, probably owing to some of the opaque material used to produce the dark background adhering to their glutinous surface (see fig. 3).

If a similar specimen is coloured with one of the ordinary bacterial stains, the stainable material is found to have diminished in size, giving the appearance of small and very irregular cocci. When the lytic action is complete, staining fails to reveal any trace of the cocci.

OBSERVATIONS ON THE PROPERTIES OF THE LYSOZYME AND ON THE CONDITIONS GOVERNING ITS ACTION.

The lysozyme is soluble in water or normal salt solution ; it is insoluble in chloroform, ether or toluol, and, as these substances do not destroy it or inhibit its action, they have been used to preserve lysozyme-containing material such as sputum for test purposes ; it retains its potency undiminished after standing at room temperature for several weeks. That lysozyme of egg white is not destroyed by desiccation, and that in the dried state it can be preserved for long periods, is shown by the fact that it is present in large amounts in commercial dried egg albumin.

From albuminous fluids, protein precipitants such as alcohol, acetone, or picric acid, precipitate the whole of the lysozyme with the proteids.

Its action takes place most rapidly when a small amount of salt is present in the fluid (under 0·1 per cent.), and ceases when more than 5 per cent. of salt is present. It acts both on living microbes and on those which have been killed with heat.

Influence of the Reaction of the Fluid.

It was found that when one drop of sputum extract and one drop of a thick suspension of *M. lysodeikticus* were added to 1 c.c. of various dilutions of hydrochloric acid or caustic soda, the lytic action was, to some extent, delayed in the tubes containing as little as 1/8,000 normal acid or 1/24,000 normal alkali, and there was complete inhibition of lysis in the tubes containing 1/800 normal acid or alkali. These figures are not strictly accurate, as alkali-free glass was not used, but they clearly indicate that the lysozyme is very sensitive to minute traces of acid or alkali.

Resistance of the Lysozyme to Heat.

Sputum extract, nasal mucus, saliva and subcutaneous fatty tissue, heated for 10 minutes at 60° C., had lost but little of their lytic power for bacteria, but 5 minutes' heat at 75° C. destroyed almost all the lysozyme. Tears, diluted 500 times with normal saline solution, were heated for 10 minutes at 75° C., and the lysozyme-content was reduced to one quarter. After boiling this dilution of tears for 30 minutes, traces of lysozyme remained active, but boiling for 1 hour apparently completely destroyed it.

In saliva, the resistance to heat of the lysozyme and of ptyalin was compared. A specimen of saliva was heated to 75° C., and specimens were taken at intervals of 1 minute, and their lysozyme and ptyalin-content were compared. It was found that these two substances disappeared from the saliva at the same time, namely, after heating for 7½ minutes.

Influence of Temperature on the Velocity of Lysozymic Action.

The lytic action takes place slowly in the ice chest and the velocity increases up to 60° C. after which it becomes slower again, probably owing to the destruction of some of the lysozyme.

If lysozyme containing material, however, is left in contact with the *Micrococcus lysodeikticus* for 24 hours the cocci are dissolved in the same dilution of the lysozyme whether the reaction takes place at room temperature, 37° C., or 50° C.

Does the Lysozyme pass through Membranes or Filters?

1. *Collodion.*—One c.c. of a saline extract of sputum was placed in a collodion sac and this was suspended in a tube containing a thick suspension of *M. lysodeikticus* and incubated for 6 hours. No lysis of the cocci took place. The sac was then punctured and the contents allowed to mix with the bacteria when complete lysis occurred within 2 minutes, showing that the sputum extract contained lysozyme, which, however, had been unable to pass through a collodion membrane.

2. *Porcelain Filter.*—Fifty c.c. of a 1 in 1,000 dilution of tears were passed through a Berkfeld filter and it was found that the filtrate was devoid of lysozyme action. As it might have been possible that some inhibitory substance (*e.g.*, acid or alkali) had been absorbed from the filter and passed into the filtrate a small quantity of the unfiltered tears was added to the tubes containing the filtrate and the cocci when lysis promptly occurred showing that the lysozyme had actually been retained on the filter and that the absence of lysis when the filtrate and cocci were mixed, was not due to the presence of any inhibitory substance.

It was impossible to obtain human secretions, rich in lysozyme, in sufficient quantity to filter them in a strong concentration through the porcelain filters available. Egg white, however, which is very rich in lysozyme, was used for this purpose in a dilution of 1 in 10 in normal saline solution. The filtrate was collected in separate portions of about 1 c.c., and each portion was tested for the presence of lysozyme. The first 19 c.c. which passed through the filter had no lytic action on *M. lysodeikticus* but after that the lysozyme passed through and in the 30th c.c. the strength of the filtrate was practically the same as that of the unfiltered material.

These experiments show that a porcelain filter is capable of absorbing a considerable quantity of lysozyme, but when that has been absorbed the filter offers no barrier to the passage of this substance. We shall see that the same thing happens with filters of cotton wool and filter paper.

Cotton Wool.—This was tested by two methods which the author had previously used to demonstrate the gossypiotropic properties of certain aniline dyes.

The first method consists in pushing slowly to the bottom of the test-tube containing a column of about 2 inches of a lysozyme-containing material a tight plug of cotton-wool, so that the fluid percolates through the cotton-wool and collects above it. Using tears diluted 1 in 1,000 (this sample of tears showed lysis up to a dilution of 1 in 5,000,000) it was found that when this experiment was carried out the whole of the lysozyme was removed by the cotton-wool.

In the second method a tight plug of cotton-wool was introduced into a narrow tube, 1 c.c. of tears (1 in 1,000) placed above this, and with pressure exerted with a rubber teat the fluid was driven through the cotton-wool. Successive volumes of 1 c.c. were driven in this way through the cotton-wool and these were separately tested for the presence of lysozyme. It was found that a tight plug of cotton-wool 1 cm. long introduced into a piece of 6-mm. tubing absorbed the whole of the lysozyme from 12 c.c. of a 1 in 1000 dilution of tears. Further volumes of the diluted tears passed through this cotton-wool plug all contained lysozyme.

Filter Paper.—This was tested in the same way as cotton-wool and with the same results. Passage through about 0·5 cm. of compressed filter paper in 6-mm. tubing removed the whole of the lysozyme from 10 c.c. of a a thousand-fold dilution of tears.

Is the Lysozyme Removed from Solution with Substances such as Charcoal?

It was found that when a small quantity of blood charcoal was added to a thousand-fold dilution of tears and after 2 hours on the bench the mixture was centrifuged, the clear supernatant fluid contained no lysozyme. It was shown that no inhibitory substance had been absorbed into the fluid from the charcoal because, after the supernatant fluid had failed to cause lysis of the cocci, a small quantity of the diluted tears was introduced, when lysis promptly occurred. It is evident, therefore, that charcoal removed the lysozyme from the fluid.

Distribution of the Lysozyme in the Body.

In the first experiments it was found that nasal mucus contained a large amount of lysozyme, and it was later found that tears and sputum were very potent in their lytic action. It was also found that this property was possessed by a very large number of the tissues and organs of the body. The lysozyme-content of the tissues was investigated by placing small portions of tissue not larger than a split pea in tubes containing 1 c.c. of a thick suspension of the *M. lysodeikticus* incubating the tubes at 45° C., and noting whether any lysis took place as evidenced by a clearing of the opacity of the suspension. Some of these tissues were obtained from the postmortem room, others from laboratory workers or from the operating theatre. The results obtained can be summed up by saying that all the tissues and organs possessed some lytic power, even a few hairs from the head causing solution of the cocci. While in these tests no attempt was made at an exact quantitative estimation, it was noticed that lysis proceeded very much more rapidly with some tissues than with others. Briefly, it may be said that epidermal structures, the lining membrane of the respiratory tract and especially the connective tissues (whether fibrous, fatty or cartilaginous) contained large amounts of lysozyme affecting *M. lysodeikticus.* The rapidity of the lysis with cartilage was so striking that an attempt was made to estimate more accurately the amount of lysozyme in this tissue. A small portion of cartilage from the patella (deep to the articular surface) was weighed and ground up in a mortar with a measured volume of salt solution. This was allowed to extract for 6 hours when it was centrifuged and the supernatant fluid was added in various dilutions to a suspension of the *M. lysodeikticus.* It was found that with an extract corresponding to one part of the original cartilage in 1,300 parts of normal salt solution, there was complete lysis of the cocci in 5 minutes at 45° C. which shows that cartilage has approximately one-tenth the lysozyme-content of tears.

The presence of lysozyme was sought for in certain physiological and pathological fluids, and the results are set forth in Table I.

Table I.

Fluids containing lysozyme.	Fluids not containing lysozyme.
Tears. Sputum. Nasal mucus. Saliva. Blood serum. Blood plasma. Peritoneal fluid. Pleural effusion. Hydrocœle fluid. Ovarian cyst fluid. Sebum. Pus from acne pustule. Sero pus from a "cold" abscess in the popliteal space. Urine containing much albumin and pus. Semen (very weak).	Normal urine. Cerebro-spinal fluid. Sweat (one sample only tested.)

In connection with the lysozyme-content of the blood, it is to be noted that, in addition to its being present in the leucocytes, in the plasma, and in the serum, it is also present in rather large amount in the fibrin of the blood clot. It is conceivable that this is a protective mechanism for open wounds, which rapidly become covered with a layer of fibrin and leucocytes, both of which are rich in lysozyme.

The lysozyme-content of tears, sputum, nasal mucus, saliva, and blood serum of the same individval were tested. The specimens were all collected at the same time and were tested about 4 hours afterwards. The titrations were carried out by making serial dilutions of the various fluids and adding to these dilutions a measured quantity of a thick suspension of *M. lysodeikticus*, after which the tubes were incubated at 45° C., and readings were made at intervals of 15, 30, and 60 minutes. The results are set out in Table II:—

Table II.—The Lysozyme-Content of various Fluids taken from the same Individual at the same Time.

Material examined.	Time of incubation at 45° C.	Dilution of fluid, 1 in :—						
	mins.		10	30	90	270	810	2430
Blood serum………	15		+	+	±	0	0	0
	30		+	+	+	±	trace	0
	60		+	+	+	+	±	0
			100	300	900	2,700		
Saliva ……………	15		+	±	0	0		
	30		+	+	±	0		
	60		+	+	±	0		
		500	1,500	4,500	13,500	40,500	121,500	
Nasal mucus………	15	+	+	+	±	±	0	
	30	+	+	+	+	±	0	
	60	+	+	+	+	±	0	
Sputum …………	15	+	+	+	±	±	0	
	30	+	+	+	+	±	0	
	60	+	+	+	+	±	0	
Tears ………………	15	+	+	+	+	0	0	
	30	+	+	+	+	±	0	
	60	+	+	+	+	+	±	

+ signifies complete clearing of the fluid.
± ,, partial ,, ,,
0 ,, no ,, ,,

It will be seen from the above Table that tears, sputum, and nasal mucus are very rich in lysozyme to the *M. lysodeikticus*, while saliva and blood serum are relatively weak. Fluids from a number of different individuals have been tested and the relative amounts of lysozyme contained in these have been found to be comparatively constant, except in the case of saliva, which seems to vary considerably, although it never approaches in lysozyme-content tears, sputum, or nasal mucus.

The Question as to whether Lysozyme exists in Tissues other than Human Tissues.

Only a limited amount of work has been done in this direction, but it is sufficient to show that lysozyme is very widespread in nature. Rabbit and guinea-pig tissues were examined and it was found that nearly all of these contained some lysozyme for the *M. lysodeikticus*, but in general the lysis was not nearly so marked as it was with the corresponding human tissues. It may be noted that the lachrymal secretion of both these animals contained no lysozyme for the *M. lysodeikticus*, against which the human tears are so powerful. The tissues of a dog were much more lytic than those of the rabbit and guinea-pig, but even they were not so active as human tissues.

It was found that egg-white was very rich in lysozyme for the *M. lysodeikticus*, there being, after incubation for 24 hours, lysis visible to the naked eye when a dilution as great as 1 in 50,000,000 was employed. Egg-white also contains lytic substances for many other bacteria. It was found also that commercial dried egg albumin was very rich in lysozyme.

In the vegetable kingdom it was found that turnip had a very definite, though not very strong lytic action on *M. lysodeikticus*. Several of the other common table vegetables were tested, but they appeared to be devoid of lytic activity.

Does the Lysozyme act on Bacteria other than the M. lysodeikticus?

In the investigation of this problem the method adopted was to make a suspension of the bacteria of such a strength that it gave a very decided opacity when diluted with an equal amount of saline; $\frac{1}{2}$ c.c. of this suspension was mixed with the same quantity of a 1-in-50 sputum extract or a dilution of tears from 1 in 100 to 1 in 1,000. As a control, a twofold dilution of the original suspension was made with normal salt solution. The tubes were incubated at 45° C. and observations were made at intervals up to 24 hours, the opacity of the tube containing sputum or tears being compared with that of the control tube.

Three groups of microbes were tested: the first group consisted of 104 strains of bacteria derived from the air of the laboratory, and of these 75 per cent. were dissolved, more or less readily, by a 1 in 100 dilution of sputum. These air-borne bacteria consisted mainly of cocci of various sorts, but there were also bacilli, yeasts, and two species of mould.

The second group consisted of a series of cultures of bacilli which are pathogenic for some animals, but not, so far as is known, for man. These were kindly supplied by Dr. St. John Brooks from the National Collection. They were tested with tears (1 in 100) and nasal mucus (1 in 100), in addition to the sputum extract, and seven out of eight cultures showed some lysis after incubation with one or other of these fluids. These cultures included *B. abortus* of Bang, and *B. pseudotuberculosis* rodentium, to both of which there was some lytic power and which will be referred to later.

The third group consisted of bacteria which had been isolated from the human body, and it was found that, whereas most of these were not acted on by the lysozyme contained in sputum or tears, some were completely, and others partially dissolved. Not one of the various members of the coli typhoid group showed the slightest signs of lysis, while sixteen out of nineteen strains of intestinal streptococci were dissolved to a greater or less extent.

The results obtained with this group of microbes are set forth in Table III, but of necessity, considering the multiplicity of strains involved, this Table is incomplete, and it may well be that by altering the conditions of the experiment somewhat, a much higher percentage of the bacteria will be dissolved.

Table III.—Effect of the Lysozyme contained in Sputum or Tears on Bacteria isolated from the Human Body,

Type of microbe.	Number tested.	Number showing some lysis.	Number showing no lysis.
Streptococci	22	16	6
Staphylococci	4	2	2
B. coli	12	0	12
B. typhosus	1	0	1
B. paratyphosus	2	0	2
B. proteus	2	0	2
B. pyocyaneus	3	0	3
B. pestis	1	0	1
M. melitensis	1	0	1
Diphtheroid Bacilli	3	0	3
Pneumococci	2	0	2

It was noticed that with different microbes, different fluids in their lysozyme-content did not always bear the same ratio to one another. Thus, while tears were apparently the most powerfully lytic to the *M. lysodeikticus*, they had a less powerful lytic effect on some other cocci than had sputum or synovial fluid. This may be the explanation of the immunity of

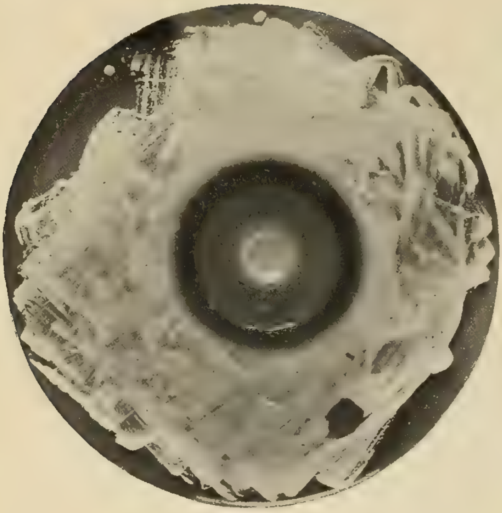

FIG. 1.

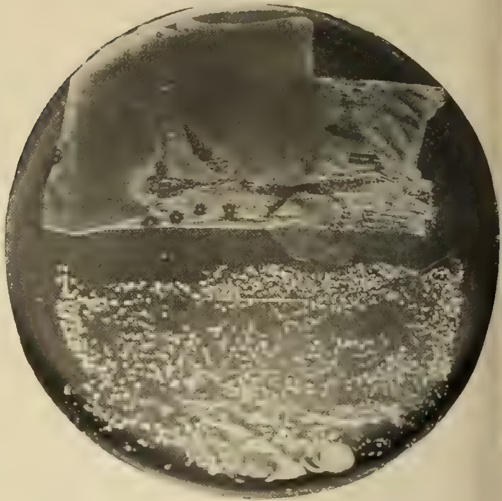

FIG. 2.

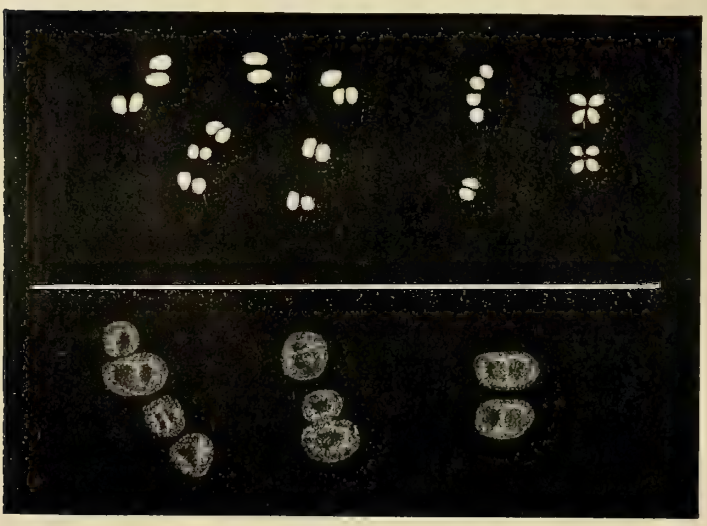

FIG. 3.

certain tissues to certain infections, or conversely the well-known predilection of certain infections for certain tissues.

The view has been generally held that the function of tears, saliva and sputum, so far as infections are concerned, was to rid the body of microbes by mechanically washing them away. Metchnikoff in his treatise on "Immunity and Infectious Disease," expresses himself very clearly and precisely on this point. From the experiments detailed above, however, it is quite clear that these secretions, together with most of the tissues of the body, have the property of destroying microbes to a very high degree.

It has not been possible to test extracts of all the different tissues to each of many microbes, but it has been shown that human tears and sputum can dissolve the majority of the microbes (presumably non-pathogenic) recovered from the air of the laboratory. Most of these air-borne bacteria are non-pathogenic, and it seems extremely unlikely that they could become pathogenic when the human secretions show such a destructive action towards them.

Again, the human secretions showed lytic power to most of the microbes tested which, although pathogenic to some animals are harmless to man. Notably there was a certain amount of lysis evident with the bacillus abortus of Bang and *B. pseudotuberculosis rodentium*, which are culturally and serologically identical with *M. melitensis* and *B. pestis* respectively, both of which latter organisms are very pathogenic for man, and for which there is apparently no lysozyme in the human secretions. It may be that it is in this sensitiveness to a human lysozyme that the difference between these microbes lies.

DESCRIPTION OF PLATE.

Fig. 1.—Photograph of agar plate with imbedded tears.

Fig. 2.—Bactericidal power of tears on streptococci. Upper half—culture from streptococci in salt solution. Lower half—culture from same number of streptococci in tears (1 in 100).

Fig. 3.—Upper half—*Micrococcus lysodeikticus* before being acted on by tears. Lower half—same partially dissolved by tears. Examined by Burri's method.

The Pigmentary Effector System. I.—*Reaction of Frog's Melanophores to Pituitary Extracts.*

By Lancelot T. Hogben, M.A., D.Sc., and Frank R. Winton, M.A.

(Communicated by Prof. E. W. MacBride, LL.D., F.R.S. Received February 2, 1922.)

(From the Zoological Laboratory, Imperial College of Science and Technology.)

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1. *Introduction.*

The ability of certain organisms, including notably the Mollusca and lower Vertebrata, to respond to their surroundings by appropriate pigmentary changes has long been familiar to biologists; it has been recognised for more than half a century that a special type of effector organs (chromatophores, melanophores, etc.) are actively instrumental in producing such changes, and that, in Vertebrates at least, pigmentary response is partially controlled through the nerves by stimuli received from the organs of vision. During the past two decades it has been shown that the reactions of the pigment cells to stimuli simulate those of other effector organs, especially as regards their responses to certain internal secretions. That a fuller understanding of their properties might prove of service to practical aspects of physiology, as well as the key to a knowledge of "colour adaptation," was realised by Lister, who concludes his paper on the cutaneous pigmentary system of the Frog (1858) with the following comment: "The pigmentary system also promises to render good service in toxicological enquiry. Hitherto in experiments performed on animals with that object attention has been directed chiefly, if not exclusively, to the effects produced upon the actions of the nervous centres, the nerves, and muscles. In the pigment cells we have a form of tissue with entirely new functions, which, though apparently allied to the most recondite processes, yet produce very obvious effects. . . . Such experiments are so readily performed, and the effects produced are so obviously indicated by the changes in colour of the integument, that I venture to recommend this method of investigation to those who are occupied in studying the action of poisons. . . ."

A considerable literature, which has been reviewed too thoroughly and recently to merit an extensive epitome here, bears witness to the intricacy of the mechanism which underlies pigmental response in Fishes, Amphibia, and Reptiles; and very little work has been done on Molluscs in this connection. It can be safely asserted that as many distinct types of chromatophores are already known as there are different sorts of other effector organs whose properties have been studied. Hence it is obvious that, apart from the intrinsic interest presented by the phenomenon of colour change, a study of the properties of this series of effectors invites consideration in relation to a fuller understanding of the *rôle* played respectively by the character of the tissue and the nature of its nerve supply in the local action of drugs. The research, of which this preliminary communication contains an initial account, of the response of Amphibian melanophores to pituitary extracts, has been approached with both these objectives in view. For present purposes it will suffice to recapitulate briefly what has already been achieved in relation to the endocrine factors in pigmental response.

Earlier workers on the pigment effector mechanism concentrated their attention on the nervous control. Corona, however, showed as early as 1898 that a glandular extract could induce pigmental changes in the frog when he brought about contraction of the pigment cells with adrenalin. Lieben (1906) confirmed this conclusion, and a similar reaction of pigment cells to adrenalin has been demonstrated in other Amphibia and in Fishes by various workers. Contraction of the melanophores of frog tadpoles was found to follow pineal administration by McCurd and F. Allen (1917), whose results have been since confirmed by Huxley and Hogben (1921). The latter have also shown that melanophore expansion follows pituitary feeding in salamander larvæ, an observation which accords with the condition of general melanophore contraction seen to follow pituitary extirpation by Allen (1916), Smith (1917), Atwell (1919), and Swingle's (1921) experiments on grafting pituitaries, published while these experiments were in progress. These workers experimented on Amphibia; Spaeth (1917), on the other hand, found that the melanophores of isolated scales in the Teleost fish *Fundulus* contract in response to both pituitary and adrenal preparations.

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Whatever may be true in the case of Fishes, this is certainly not the mode of response among Amphibia, as the present experiments are intended to indicate. Pituitary extracts injected into the frog produce a visible darkening, with complete and extreme expansion of the melanophores. The results recorded show that this is a specific reaction of extracts of the posterior lobe, is produced by a principle probably identical with the uterine stimulant, and is independent of the nerve-endings in the local effect which it evokes.

The normal reactions of melanophores to light in the frog depend on several factors besides light, temperature being of predominant significance. For the purpose of the experiments, it was necessary to use frogs in which the melanophores were fully contracted. Decerebrated animals were employed: the room temperature was uniformly 73° to 77° F. At this degree of warmth, the frogs placed on a white background under bright illumination show complete contraction of the melanophores, which condition persists after the animals have been decerebrated carefully, so as to avoid injury to the pituitary of the animal itself. Throughout this paper, the melanophores referred to are in all cases the *dermal* melanophores.

2. *Specificity and Localisation of the Pituitary Melanophore Stimulant.*

The experiments recorded in subsequent sections were based upon pituitary extracts prepared commercially for clinical purposes. Such preparations are made from the posterior lobe of the gland, which includes not only the infundibulum *sensu stricto*, *i.e.*, the pars nervosa, but, in addition, the pars intermedia, which is ontogenetically a hypophysial structure, though the term hypophysis is sometimes inaccurately applied to the whole gland or to the pars anterior (anterior lobe) alone. Hence the term infundibulin applied to such extracts is misleading. The following preliminary experiments indicate the existence of a specific melanophore stimulant in pituitary extracts and its localisation in the pars intermedia and nervosa :—

(i) An adult female rabbit was decapitated, and its pituitary gland, together with pieces of muscle, brain, ovary, pancreas, suprarenals and spleen removed at once. The tissues were severally weighed, ground up with sand, made up to 1 per cent. in Ringer, and left in the thermostat at 35° C. for 2 hours. At the end of 2 hours eight pairs of pale frogs were taken, being injected in pairs (0·5 c.c. per individual), with the extracts of tissues enumerated, another pair being injected with a 0·1 per cent. solution of pituitary, and an additional pair with an extract of putrid meat. The object of the latter injection was to control the possibility that the effects found to follow injections of commercial products were not due to traces of the physiologically active (pressor) substance known to occur in putrefying tissues. After half an hour, the pairs injected with 1 per cent. and 0·1 per cent. pituitary extracts had assumed a coal-black hue, while all the others remained pale: microscopic examination of the skin showed that this effect was due to the expansion of the melanophores in the former case.

(ii) The small size of the pituitary gland in the rabbit as well as the fact that the rabbit's pituitary is remarkably compact, having hardly any cleft between the two lobes (which are thus additionally difficult to separate), made it impossible to test the effect of extracts from different parts of the gland in the foregoing experiment. In the case of the ox pituitary the demarcation between the three parts is very striking. It was possible to secure ox pituitaries from the slaughter house within little more than an hour of killing, and to dissect away portions of the pars nervosa, intermedia and anterior separately, for preparing extracts of the three divisions of the gland. Each portion was weighed, ground up with sand, and after extraction with Ringer at 35° C. for 2 hours made up to a 0·1 per cent. and 0·02 per cent. solution. A pair of pale frogs was injected with each of the six solutions (0·5 c.c. per individual). After twenty minutes the four frogs injected with anterior lobe extract showed no darkening. The pair injected with weak (0·02 per cent.) pars nervosa extract likewise displayed no darkening, whereas both frogs which had been injected with 0·1 per cent. pars nervosa extract and all four animals injected with the pars intermedia preparation (0·1 per cent. and 0·02 per cent.) showed intense darkening of the skin. Microscopic examination showed that the three pairs injected respectively with pars anterior, strong and weak extracts, and pars nervosa weak extract, had the melanophores in the contracted condition, while the remaining six, those injected with pars intermedia and pars nervosa strong extract, displayed a state of general melanophore expansion of the characteristic type.

From these experiments it is clear that the pituitary gland contains a specific principle which is capable of inducing an extreme type of melanophore expansion in the frog, and that the production of this substance is located in the posterior lobe. It will be noted that the extract prepared from the pars intermedia was in the last experiment more potent than that prepared from the pars nervosa in corresponding amounts of the fresh glandular substance. The fact that workers like Swingle, who record the effect of grafting experiments, claim for the pars intermedia an exclusive *rôle* in the pigmental control of the pituitary gland, does not necessarily conflict with this result, for it is on histological grounds unlikely that the pars nervosa actually secretes the autocoid which produces melanophore expansion. What is most likely, as has been suggested by other writers, is that the pars intermedia secretion rapidly diffuses into the nervosa, in which case the term infundibulin applied to such extracts is not merely confusing but positively incorrect. The changes which follow injection of pituitary extracts in the frog confirm the conclusion that the condition of pallor which results from pituitary removal in amphibian larvæ, as shown by Allen and others, is due to endocrine deficiency.

3. *Sensitiveness of the Melanophores to Pituitary Extracts.*

The following tests indicate the mode of response of frogs' melanophores to different concentrations of pituitary extracts. Owing to the extreme simplicity of the technique employed, it is not desirable to repeat the description where experiments have been repeated for confirmatory purposes. The extract employed was in all the remaining experiments Burroughs and Wellcome's liquid sterile posterior lobe extract ("infundin") in 0·5 c.c. tubes.

(1) Solutions 0·5 per cent, 0·05 per cent., 0·005 per cent., 0·0005 per cent. of the liquid extract in frog's Ringer were made up, 1 c.c. of each solution being injected into each of a pair of pale frogs; a fifth pair was injected with saline simultaneously as a control. At the conclusion of half an hour (by which time maximum reaction is attained with pituitary extracts), the two pairs injected respectively with 0·5 per cent. and 0·05 per cent., showed the striking pigmentary changes described in the last section; the remainder were still pale. On removing a piece of skin from the back of each frog, fixing in Bouin's fluid, dehydrating and mounting in balsam, it was seen that in the two pairs that showed visible darkening, the melanophores were so fully expanded that their processes appeared to form a continuous web, thus rendering the skin almost opaque. The other frogs showed complete contraction of the melanophores, except in the 0·005 per cent. pair, in which there was perhaps a very slight tendency towards a stellate condition of the melanophores. The visible darkening of the skin appears within 10 to 20 minutes, reaches its maximum in half an hour, and disappears within 3 hours from the time when injection takes place, as was shown by a further injection of the 0·5 per cent. solution into another pair of animals. Pieces of skin were also placed in the stronger pituitary solution to test its action on the isolated skin. So treated, they displayed on microscopic examination half an hour later, complete expansion of the melanophores, while other pieces placed in saline showed the melanophores contracted to fine points. It should be explained, however, that if the skin is subjected to a good deal of mechanical stimulation, as for example, after being cut into small strips 2 mm. square, the melanophores remain contracted and will not respond by expansion to pituitary extract.

(2) A second tube of infundin was used to make up solutions, 0·1 per cent., 0·075 per cent., 0·05 per cent., 0·025 per cent., 0·01 per cent. and 0·005 per cent. Six pairs of frogs were taken and injected (0·5 per cent. per individual)

severally with the above strengths. The first and fifth doses were, it will be noted, quantitatively equivalent to the second and third in the foregoing experiment. At the end of half an hour the first two pairs (0·1 per cent. and 0·075 per cent.) were intensely dark; the others showed but slight, if any, perceptible change. Microscopic examination of pieces of the skin showed that in the first two pairs the melanophores were expanded, whereas in the remainder they were contracted. It may be well to observe parenthetically in this connection, that the terms *contraction* and *expansion* are used in a purely descriptive sense throughout this paper, without prejudice to the controversial issue, to be dealt with subsequently it is hoped, whether the pigment cell as a whole contracts and expands, or whether on the other hand the appearance is due to the independent migration of the pigment granules.

The above experiments illustrate two significant aspects of the pituitary melanophore reaction: (*a*) there is a fairly definite end point; and (*b*) it is extremely sensitive when the clinical dose is taken as a basis of comparison. Since 0·5 to 1 c.c. are usually administered clinically (*e.g.*, in parturition), it will be seen that less than a thousandth of the clinical dose, or 0·0004 c.c. of the commercial (21 per cent.) extract, suffice to evoke the response.*

To obtain more crucial data it will be necessary to substitute for the crude method of injection *viâ* the dorsal lymph sac the intravenous or intraperitoneal operation. Considering the extreme technical facility of this method of testing the activity of pituitary preparations, the rapidity with which it can be carried out, the simplicity and cheapness of the materials employed and finally the probable identity—as will be seen later—of the melanophore and uterine stimulants, it is not premature to suggest in this place that the pituitary melanophore reaction merits the fullest consideration as an alternative to the very elaborate methods now employed for the standardisation of clinical preparations. It would be well moreover to explore the possibility of employing strips of frog skin as Hooker was successful in culturing the latter. It should then be possible to eliminate the factor of individual variation as a source of error. Spaeth emphasised this point in relation to the use of fish scales: the frog has the merit of being much more easily obtained and reared; and since excitement and mechanical stimulation ordinarily cause the frog's pigment cells to contract, everything favours the success of manipulation.

4. *Relation of the Melanophore Stimulant to other Pituitary Autocoids.*

It is now generally recognised that the diverse responses evoked by extracts of the posterior lobe of the pituitary gland are not due to a single autocoid.

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* 0·21 gr. fresh infundibular substance in 1 c.c.

By extraction of dried and powdered infundibulum with acidified water, treatment of the solution with colloidal ferric hydroxide and subsequent continuous extraction of the filtrate at reduced pressure with butyl alcohol, Dudley (1919) succeeded in separating a crystalline residue containing all the uterine stimulant (oxytocic principle) together with a portion of the pressor substance. The latter is again differentiated from the uterine stimulant by the fact that it is rapidly and completely destroyed by boiling with 0·5 per cent. HCl: on the other hand one-fifth of the uterine stimulant remains after half-an-hour of acid hydrolysis, and at the end of 6 hours a slight trace—less than 1/200ths persists. The rapid destruction of the pressor principle was shown by Abel and Nagayama (1920) whose results have been confirmed by Dale and Dudley (1921), and extended as indicated above. There are, therefore, at least two active principles in the extracts of the infundibulum; and the question thus arises of what relation exists between the melanophore stimulant of the infundibulum and the other pituitary autocoids.

The effect of continued acid hydrolysis was investigated as follows. A 0·5 per cent. solution of the commercial extract was made up in 0·5 per cent. HCl. This mixture was subjected to continuous boiling for 5 hours, a sample being removed at the end of 30 minutes. At the conclusion of the experiment a sample of the unboiled mixture, the portion which had been subjected to only half-an-hour's hydrolysis, and the residue were respectively neutralised with soda and diluted to a concentration approximately isotonic with Frog's Ringer. From each of the three solutions, A (unboiled), B (boiled half-an-hour), C (boiled 5 hours) 0·5 c.c. was injected into a pair of frogs whose pigment cells were fully contracted. The macroscopic and microscopic examination of the six animals at the conclusion of an hour revealed a marked contrast. The A and B pairs were dark and showed the typical pituitary reaction. The pair C remained pale. Microscopic preparations of the skin showed that in the C pair the melanophores were fully contracted, and in the A pair displayed extreme expansion. Those of the B pair were not so extremely expanded as those of the A pair. The result of the experiment indicates that pituitary extracts retain a considerable potency to induce melanophore response after half-an-hour's boiling with 0·5 per cent. HCl. Hence the melanophore stimulant is not identical with the pressor substance, and in its slow destruction by acid hydrolysis behaves in a manner identical with the uterine or oxytocic principle of infundibular extracts, as far as is demonstrable without extensive quantitative estimation of the potency of each sample.

5. *Relation of the Melanophore Stimulant to Histamine.*

The possibility that the melanophore stimulant present in infundibular extracts is identical with the oxytocic principle led to experiments on the effect of histamine, since Abel and Kubota (1919) have raised the question of the identity of histamine and the uterine stimulant. Dudley has since produced evidence that the effects of pituitary extract on plain muscle are not due to the presence of traces of histamine as these workers suggested, and more recently Dale and Dudley (1921) have conclusively shown that Abel and Kubota were wrong on this point. It can also be stated, with the utmost confidence, that the melanophore stimulant is likewise not identical with histamine; doses of the latter as considerable as 0·0036 grm. do *not* induce melanophore expansion in the frog.

That the two substances are not identical is shown further by the action of trypsin. Histamine is not acted upon by trypsin,* whereas Dale (1909) and Dudley (1919) have shown that both the commonly recognised pituitary autocoids are destroyed rapidly by trypsin. In this connection it may be noted that Schafer and Herring (1906) claimed that pepsin destroys the pressor activity of pituitary extract, leaving intact the diuretic principle; they denied that trypsin destroyed either. The following experiment indicates that pepsin does not affect the melanophore stimulant, but that the latter is rapidly destroyed by trypsin.

Six solutions were made up as follows:—

A. 0·5 per cent. infundibular extract in 0·2 per cent. HCl and 0·5 per cent. pepsin.

B. 0·5 per cent. infundibular extract in 0·2 per cent. HCl, without pepsin.

C. 0·2 per cent. HCl and 0·5 per cent. pepsin.

D. 0·5 per cent. infundibular extract in 0·5 per cent. saline trypsin.

E. 0·5 per cent. saline trypsin alone.

F. 0·5 per cent. infundibular extract in 0·5 per cent. boiled solution of trypsin.

At the conclusion of 2 hours' digestion at 34° C. A–C were neutralised, boiled, and diluted to a concentration approximately isotonic with Ringer. D–F were boiled. After cooling, 0·5 c.c. of each fluid was injected into a pair of frogs; and the 12 animals were examined 45 minutes afterwards, a strip of skin being removed for microscopic examination.

Of A–C both the A and B pairs showed macroscopically and microscopically the characteristic pituitary reaction. Of D–F, F alone showed expansion of the melanophores on being examined by macroscopic and microscopic methods.

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* Dudley (*op. cit.*).

The result of the experiment indicates that the melanophore stimulant of pituitary extracts is not destroyed by pepsin or HCl 0·2 per cent., but that it is completely and rapidly destroyed by trypsin. This is in accordance with Dale's work on the uterine and pressor principles.

As the foregoing experiments show, the melanophore stimulant, like the other pituitary autocoids, is not destroyed by boiling; it is immediately destroyed in the presence of a small quantity of H_2O_2. One other point deserves mention in this connection to correct a possibly erroneous interpretation of results previously published by one of the authors. It was stated (Huxley and Hogben, 1921) that pituitary feeding induces melanophore expansion in salamander larvæ. In view of the destruction of the melanophore stimulant by trypsin, it seems highly probable that the effect was not produced as in the case of thyroid feeding by absorption of the autocoid *viâ* the digestive system, but by traces in solution in the medium, acting through absorption by the skin.

6. *Mode of Action of the Melanophore Stimulant.*

The effect of injection of pituitary extracts on the intact animal might be interpreted in at least four different ways:—

(*a*) Local effect on the circulation (vasomotor).
(*b*) Central stimulation.
(*c*) Action on nerve-endings in melanophores.
(*d*) Direct action on the melanophores.

The first two are dismissed in view of the fact that, with appropriate preparations, pituitary extracts can be made to exert their characteristic reaction upon isolated skin. The second is also excluded by the possibility of producing the same reaction after destruction of both spinal cord and brain. To discriminate between (*c*) and (*d*), two methods may be adopted: first, the injection of pituitary extract after the complete degeneration of the nerve supply of the skin in a particular area; and, secondly, injection after paralysis of the nerve-endings which supply the melanophores by means of the usual drug series. The first is the more critical, and it is hoped to carry out this method later. The second is open to the objection that the evidence is based on the analogy of the operation of drugs on plain muscle. As far as the results of experiments on these lines permit legitimate inference, it would appear that the action of the infundibular melanophore stimulant is direct and independent of any nerve-endings.

The following re-agents were used for paralysis of different types of nerve-endings: cocaine (afferent), curare (spinal efferent), atropine (parasympathetic), and apocodeine (sympathetic). The use of cocaine was suggested by the possibility of an antidromic control. The subsequent action of pituitary extract was tested at varying intervals following doses of different magnitude, to make certain both as regards time of action and amount of drug administered, that the full effect of the latter would be exerted. In no case did the normal response fail to make its appearance. The indications are therefore that the pituitary melanophore stimulant acts directly on the melanophores of the frog and not on the nerve-endings, as is, indeed, fully consonant with what is at present known of the mode of action of the other pituitary autocoids.

Neither curare, atropine nor cocaine affect the melanophores of the frog in any way. While injection evoked its maximum effect in decerebrated frogs which had received just over the normal dose of curare adequate to produce general motor paralysis, it should be noted that very large doses of curare did prevent the melanophore response to pituitary injection before the circulation had actually ceased. Repeated tests, with a wide range of doses, showed that atropine does not cause the melanophores of the frog to expand as do those of the fish *Fundulus* when so treated (Spaeth). Laurens (1915) has also noted the inefficacy of atropine to induce expansion in the contracted melanophores of the Axolotl. The different mode of reaction of the dermal melanophores of Fishes and Amphibia towards both atropine and the pituitary autocoid would suggest that the term "dermal melanophore" has been applied to more than one type of effector organ. It is intended to reserve, for fuller discussion at a later stage, the reactions of frog melanophores to drugs; but it may here be mentioned, in connection with the antagonism of atropine and pilocarpine in respectively paralysing and stimulating parasympathetic and secretory nerve-endings, that the latter re-agent does not induce expansion of the contracted melanophores in the frog. Were these structures innervated by the parasympathetic it would be expected that either one or the other would operate in this way. Apocodeine which, as Dixon (1904) has shown, effects general sympathetic paralysis, brings about a darkening of the skin, not sufficient, however, to mask the subsequent effect of pituitary administration. This is fully consonant with the reaction of the melanophores to adrenalin, and the direct evidence given by Hooker (1912) in regard to the relation of sympathetic stimulation to melanophore contraction.

Finally, the reaction studied in the experiments here recorded, leads to the possibility that the nervous system may not be such an all-important factor in the mechanism of "colour adaptation" as it has been customary to believe in the past. Laurens (1915) concludes from his experiments on the pigment responses of blinded and seeing axolotls, that in "colour adaptation" among the Amphibia the stimuli received by the retina and transmitted to the central nervous system are "indisputably" there "transformed and sent out along motor nerves to the pigment cells." That the retina exerts a controlling influence is admitted; that the stimuli it receives are transmitted to the central nervous system can hardly be questioned; but that the control of the pigment cells thereby is of a purely reflex character cannot be conceded till the endocrine factor has been eliminated. The existence of an endocrine substance capable of producing the reverse response to adrenalin,* raises the possibility that the central nervous system may exercise its control over the pigment cells partly at least through the secretory activity of the suprarenal and pituitary glands.

7. *Summary.*

1. Extracts of the posterior lobe of the pituitary gland have a specific effect on the melanophores of the frog causing them to undergo extreme expansion; an injection, equivalent to less than 1/1000th of the ordinary clinical dose, is adequate to produce a conspicuous darkening of the skin visible to the naked eye. The effect of pituitary extract on the dermal melanophores of the frog is thus the reverse of that which follows administration of adrenalin in the frog, and both adrenal and pituitary autocoids in Fundulus, if Spaeth's observations on the latter are correct.

2. The melanophore stimulant of pituitary extracts is only slowly destroyed by boiling with 0·5 per cent. HCl; it is hence not identical with the pressor principle, and in its slow destruction by acid hydrolysis agrees with the oxytocic or uterine principle.

3. That it is not identical with histamine is shown both by the inefficacy of this drug to induce expansion of the melanophores, and by the readiness with which it is destroyed by tryptic digestion; it is not destroyed in a corresponding manner by pepsin.

4. That the pituitary stimulant acts directly on the dermal melanophores rather than on the nerve endings is indicated by the failure of apocodeine, atropine, curare, and cocaine, to abolish the reaction when administered in doses which, on general grounds, would be regarded as sufficient to paralyse all nerve endings.

This research was carried out in Prof. McBride's laboratory; acknowledgment is made to Prof. McBride for his kindness in reading the MS., and to Dr. H. H. Dale for generous assistance with reference to the literature.

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* *Cf.* Introduction.

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*Relationships between Antiseptic Action and Chemical Constitution with special reference to Compounds of the Pyridine, Quinoline, Acridine and Phenazine Series.**

By C. H. Browning, J. B. Cohen, F.R.S., R. Gaunt, and R. Gulbransen.

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(From the Organic Chemistry Laboratory, University of Leeds, the Bland-Sutton Institute of Pathology, Middlesex Hospital, and the Pathological Department of the University and Western Infirmary, Glasgow.)

Biological Section.

In the course of investigations on the antiseptic properties of certain basic benzene derivatives, it was shown that the methochloride of diaminoacridine was very highly antiseptic, and that, unlike other powerful antiseptics then known, its antibacterial activity was not reduced by the presence of blood serum (Browning, Gilmour, Gulbransen, Kennaway and Thornton, 2, 7). Diaminoacridine methochloride had been prepared by

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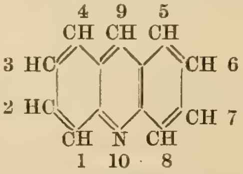
* The work reported in this communication was done with the support of the Medical Research Council.

Benda (1) for Ehrlich, and was named by them trypaflavin on account of its powerful therapeutic properties in experimental trypanosome infections. We are not aware that its action on bacteria had been investigated prior to the work recorded in the above paper (2), although Shiga (10) published almost simultaneously results of his investigations on its action on *V. choleræ.* The hydrochloride and sulphate of diaminoacridine were then found to be practically equal to the methochloride in antiseptic properties and in efficacy of action in serum (8). In the case of these aminoacridine substances the power of killing bacteria is exerted slowly; thus, with the methochloride acting on *B. coli* in serum, the concentration which proves lethal in 2 hours (1 : 20000) is five to ten times greater than that required to produce sterility after 24 hours; on the other hand, mercuric chloride and carbolic acid produce their maximum effect within 2 hours, whether they be tested in a solution containing a minute amount of protein (0·7 per cent. of Witte's peptone) or in a rich protein medium such as serum. The acridine compounds, therefore, may be said to act especially as bacteriostatic agents (7, 8). This property, coupled with their relative innocuousness for mammalian tissues, as tested by toxicity for the living animal as a whole (4, 7), effect on phagocytosis (3, 7), and slight irritating action on the conjunctiva (7), suggested that these substances should be specially applicable for the purpose of restraining bacterial infections in the tissues. Thus the methochloride, under the name of acriflavine, and the sulphate of diaminoacridine base, as proflavine, found extensive use in the treatment of infected wounds during the late war, and also in the treatment of such relatively accessible infections as gonorrhœa. The present work records the investigations which have been made with the view of tracing the source of the antiseptic property of diaminoacridine compounds by examining substances which may be regarded as fragments of the acridine molecule. The parent substance of the acridine derivatives is a compound of the following formula :—



Acridine.

that is, a combination of two benzene and a pyridine ring. If the two side wings are removed, a pyridine nucleus remains; if only one wing is detached, a quinoline nucleus results. It seemed, therefore, possible that the antiseptic activity of acriflavine (diamino acridine methochloride) might reside either in the pyridine or quinoline nucleus, reinforced by one or more amino groups. It is for this reason that substances of this type were first examined.

In addition, a series of acridine derivatives have been prepared and tested for their antiseptic power in order to determine, if possible, whether any law could be established relating chemical structure and antiseptic action within the group. Also, observations have been made upon phenazine compounds, on account of their close chemical relationship to the acridine group.

Methods of Estimating Antiseptic Power.

The substance to be tested, in a volume not exceeding 0·1 c.c., was added to 1 c.c. of the culture medium, which consisted: (*a*) of a watery solution containing 0·35 per cent. sodium chloride, and 0·7 per cent. bacteriological peptone, such as Witte's, the hydrogen-ion concentration of the mixture being adjusted by the addition of caustic soda to yield a P_H value between 7·2 and 7·8 as indicated by the usual indicators; and (*b*) sterile ox serum, which had been previously heated for several hours at 56° C., in order to destroy normal bactericidal power as well as accidental contaminating organisms. Serum, in virtue of its content in protein, has a powerful action in reducing the bactericidal effect of most strong antiseptics, at the same time it represents the fluid constituent to which antiseptics in contact with the tissues are exposed, *e.g.*, in a surgically treated wound; serum also acts as a satisfactory culture medium for the two types of organisms employed, and is extremely constant in composition and reaction. Hence, serum may be regarded as a highly suitable medium in which to test antiseptic action. The organisms employed in the tests were *Staphylococcus aureus* and a single strain of *B. coli*; the inoculation dose being 0·1 c.c. of a 1 : 1000 dilution in saline of a 24 hours' culture in peptone water. Experiments have shown that within wide limits the efficiency of the antiseptic, as tested by the method described, is practically independent of the size of the inoculum (3, 8). But inoculation with very large numbers of organisms should be avoided, as these fail to maintain themselves in the medium even in the absence of any antiseptic (3). In general, the following series of concentrations of each substance was tested, 1 : 1000000, 1 : 400000, 1 : 200000, 1 : 100000, 1 : 40000, 1 : 20000, 1 : 10000, 1 : 4000, 1 : 2000, 1 : 1000. Thus, in examining any given compound, the effect of varying concentrations was tested at the same time and with the same batch of medium and the same cultures of organisms. The mixtures were incubated at 37° C. for 48 hours, and then the final readings were made; the occurrence of abundant growth was shown by the development of turbidity in the previously clear medium, but subcultures frequently yielded growth from tubes which appeared to be clear to the naked eye. Accordingly, in all cases the presence or absence of living organisms was decided by subculturing each mixture on nutrient agar, which was then incubated for 48 hours at 37° C. The results are recorded numerically, the highest concentration which permitted vigorous growth, and the lowest producing sterility, as tested by subculture of a loopful of the mixture on agar, being given. In certain cases there is a wide zone separating these two concentrations, which indicates that the particular compounds are specially bacteriostatic in action, and that concentrations considerably less than that required to kill the bacteria still have the effect of restraining growth. It is to be noted that more useful information is obtained by making subcultures from the mixtures of antiseptic and organisms on a solid medium than in a fluid one, as in the latter case it is not possible to determine any degree of action of the antiseptic short of complete sterilisation. The investigation has been complicated by such questions as effects due to differences in solubility and variations in dissociation, leading possibly to differences in hydrogen-ion concentration of the solutions. Thus it has been found, when examining particular compounds, that comparatively small variations in hydrogen-ion concentration may exercise a great influence on the antiseptic potency (Browning, Gulbransen and Kennaway, 6). With diaminoacridine methochloride in peptone water, the concentration required to sterilise *B. coli* when the P_H value of the solution lay between 4 and 5 was 1 : 2000; within a range from 6 to 7, 1 : 10000 of the dye sufficed; at 8 to 9, 1 : 40000 sterilised, while at 11 a concentration of 1 : 200000 was sufficient. In each case the medium with similar reaction, but without the antiseptic, permitted vigorous growth of the organisms. In addition, there is the further factor of variability in the behaviour of the bacterial culture. With regard to the latter, it appears to be highly probable, if not definitely established, that the individuals in a given culture are not all equally susceptible to harmful influences; thus, irregularities are observed when a particular concentration of antiseptic is caused to act on duplicate samples of the same infected material. This has been drawn attention to by Richet and Cardot (9), and has been observed also in our own work. Further, the occurrence of variations in the culture from time to time can scarcely be excluded although no evidence has been obtained pointing to permanent or to regularly cyclic changes. Repeated series of tests carried out with a view to examining the action of diaminoacridine methochloride on a single strain of *B. coli* in ox serum have shown the following variable results. A concentration of 1 : 1000000 and upwards, sterilised in 8 series, 1 : 400000 and upwards, sterilised in 17 series, 1 : 200000 and upwards in 16 series, 1 : 100000 and upwards, in 24 series, 1 : 40000 in 2 series, and 1 : 40000 failed to sterilise in 1 series. With *Staphylococcus aureus*, however, the range of variation was distinctly less. Accordingly, the numerical values recorded here must be interpreted in the light of the above results (5). It should be noted, however, that in comparing certain closely related compounds in which marked alterations in antiseptic power may be produced by relatively slight chemical differences, *e.g.*, the methochloride and the hydrochloride of the same base, the two substances have been tested on the same occasion and with the same specimen of medium and culture, thereby reducing as far as possible the action of uncontrollable factors.

Fragments of the Acridine Molecule.

Table I includes all the compounds tested, and comprises pyridine and quinoline derivatives and dinaphthylimine. The striking feature, in general, is the low grade of antiseptic power shown by these bodies. Thus, the hydrochlorides of quinoline [3], tetrahydroquinoline [15], and the aminoquinolines (*o* [4], *m* [6], *p* [8], and *a* [10]), all failed to sterilise in dilutions exceeding 1 : 2000 either in peptone water or in serum. The methochlorides of the aminoquinolines [7, 9, 11], except in the case of the ortho-compound [5], showed accentuation of antiseptic action in serum, as compared with the hydrochlorides of the corresponding bases, a characteristic result which will be discussed in more detail when dealing with the acridine group. The hydrochlorides of α- [17] and β-naphthoquinoline [19] were slightly more active.* No striking difference could be established between these and their tetrahydro-derivatives [22, 23]. Diamino β-naphthoquinoline [24] also showed no enhanced efficiency. The methochlorides (or methosulphates) of both naphthoquinolines [18, 20, 21] and of diamino-β-naphthoquinoline [25] showed intensified action in serum. The 8-hydroxyquinoline sulphate [12], long known as an antiseptic under the name of "chinosol," is included for comparison; its activity for *Staphylococcus aureus* contrasts with the slight effect of hydroxyacridine compounds [67] and of the aminoquinoline compounds as antiseptics, but it is very weakly antiseptic for *B. coli*. On the other hand, it is remarkable that the methochloride [13] and methopicrate [14] of the base do not show enhanced action. 1·1·dinaphthyl-2·2·imine [28] exhibits great discrepancy between its powerful action on *staphylococcus* and lack of effect on *B. coli*, which is similar to that exhibited by the triaminotriphenylmethane compounds, hexa-methyl and ethyl-violet. But the most striking character of this substance is the reduction in action produced by

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* Previous results recorded for α- and β-naphthoquinoline were obtained with less pure preparations (see 'Journal of Pathology and Bacteriology,' vol. 24, p. 127 (1921)).

Table I.—The Antiseptic Action of Fragments of the Acridine Molecule—Quinoline and Pyridine Derivatives and Dinaphthylimine.

(In this and subsequent Tables + indicates that free growth of the organisms occurred in the concentration of substances mentioned, while — indicates a sterile mixture; *inh.* denotes inhibition of growth short of complete sterilisation. *Ppt.* indicates that precipitation has occurred in the mixture of medium and chemical compound.)

No.	Substance.	Organism.			
		Staphylococcus aureus : medium		*B. coli* : medium	
		Peptone water.	Serum.	Peptone water.	Serum.
1	*a*-aminopyridine hydrochloride	1 : 4000 + ? —	1 : 1000 + ? —	1 : 1000 + ? —	1 : 1000 + ? —
2	*a*-dimethylaminopyridine methiodide	1 : 20000 + 1 : 2000 —	1 : 10000 + ? —	1 : 1000 + ? —	1 : 100000 + ? —
3	Quinoline hydrochloride	1 : 4000 + 1 : 2000 —	1 : 1000 + ? —	1 : 2000 + 1 : 1000 —	1 : 1000 + ? —
4	*o*-aminoquinoline hydrochloride	1 : 4000 + 1 : 2000 —	1 : 1000 + ? —	1 : 1000 + ? —	1 : 1000 + ? —
5	*o*-aminoquinoline methochloride	1 : 4000 + 1 : 2000 —	1 : 1000 + ? —	1 : 1000 + ? —	1 : 1000 + ? —
6	*m*-aminoquinoline hydrochloride	1 : 2000 + 1 : 1000 —	1 : 1000 + ? —	1 : 1000 + ? —	1 : 1000 + ? —
7	*m*-aminoquiniline methochloride	1 : 4000 + 1 : 1000 (*inh.*)	1 : 10000 + 1 : 4000 —	1 : 1000 + ? —	1 : 10000 + 1 : 4000 —
8	*p*-aminoquinoline hydrochloride	1 : 1000 + ? —	1 : 1000 + ? —	1 : 1000 + ? —	1 : 1000 + ? —
9	*p*-aminoquinoline methochloride	1 : 2000 + 1 : 1000 (*inh.*)	1 : 10000 + 1 : 2000 —	1 : 1000 + ? —	1 : 40000 + 1 : 4000 —
10	*a*-aminoquinoline hydrochloride	1 : 4000 + 1 : 1000 (*inh.*)	1 : 1000 + ? —	1 : 1000 + ? —	1 : 1000 + ? —
11	*a*-aminoquinoline methochloride	1 : 1000 + ? —	1 : 10000 + 1 : 4000 —	1 : 1000 + ? —	1 : 10000 + 1 : 4000 —

12	8-hydroxyquinoline sulphate ("chinosol")	1 : 2000000 +	1 : 400000 +	1 : 40000 +	1 : 40000 +
		1 : 400000 —	1 : 100000 —	1 : 1000 —	1 : 1000 —
13	8-hydroxyquinoline methochloride	1 : 400000 +	1 : 20000 +	1 : 10000 +	1 : 4000 +
		1 : 100000 —	1 : 10000 —	1 : 1000 —	1 : 1000 —
14	8-hydroxyquinoline methopicrate	1 : 100000 +	1 : 20000 +	1 : 2000 +	1 : 4000 +
		1 : 40000 —	1 : 4000 —	1 : 1000 —	1 : 2000 —
15	Tetrahydroquinoline hydrochloride	1 : 2000 +	1 : 1000 +	1 : 1000 +	1 : 1000 +
		1 : 1000 —	? —	? —	? —
16	Methyl-tetrahydroquinoline methiodide	1 : 1000 +	1 : 1000 + (*ppt.*)	1 : 1000 +	1 : 1000 + (*ppt.*)
		? —	? —	? —	? —
17	*α*-naphthoquinoline hydrochloride	1 : 10000 +	1 : 2000 +	1 : 2000 + (*ppt.*)	1 : 1000 +
		1 : 4000 —	1 : 1000 (*inh.*)	1 : 1000 — (*ppt.*)	? —
18	*α*-naphthoquinoline methosulphate	1 : 20000 +	1 : 40000 +	1 : 4000 +	1 : 100000 +
		1 : 4000 —	1 : 10000 —	1 : 1000 —	1 : 40000 —
19	*β*-naphthoquinoline hydrochloride	1 : 10000 +	1 : 2000 +	1 : 10000 +	1 : 1000 +
		1 : 4000 — (*ppt.*)	1 : 1000 (*inh.*)	1 : 4000 — (*ppt.*)	? —
20	*β*-naphthoquinoline methochloride	1 : 20000 +	1 : 20000 +	1 : 2000 +	1 : 100000 +
		1 : 10000 —	1 : 10000 —	1 : 1000 —	1 : 10000 —
21	*β*-naphthoquinoline methosulphate	1 : 40000 +	1 : 20000 +	1 : 1000 +	1 : 40000 +
		1 : 4000 —	1 : 10000 —	? —	1 : 4000 —
22	Tetrahydro-*α*-naphthoquinoline hydrochloride	1 : 10000 + (*ppt.*)	1 : 1000 +	1 : 10000 + (*ppt.*)	1 : 1000 +
		1 : 4000 — (*ppt.*)	? —	1 : 4000 — (*ppt.*)	? —
23	Tetrahydro-*β*-naphthoquinoline hydrochloride	1 : 10000 +	1 : 2000 +	1 : 4000 + (*ppt.*)	1 : 1000 +
		1 : 2000 — (*ppt.*)	1 : 1000 (*inh.*)	1 : 2000 — (*ppt.*)	? —
24	Diamino-*β*-naphthoquinoline*	1 : 4000 +	1 : 4000 +	1 : 2000 +	1 : 1000 +
		1 : 2000 —	1 : 1000 —	1 : 1000 (*inh.*)	? —
25	Diamino-*β*-naphthoquinoline methochloride	1 : 2000 +	1 : 10000 +	1 : 1000 +	1 : 10000 +
		1 : 1000 —	1 : 4000 —	? —	1 : 4000 —
26	1.4.naphtho-dipyridine hydrochloride†	1 : 4000 + (*ppt.*)	1 : 1000 + (*ppt.*)	1 : 4000 + (*ppt.*)	1 : 1000 + (*ppt.*)
		1 : 1000 — (*ppt.*)	? —	1 : 2000 — (*ppt.*)	? —
27	1.5.naphtho-dipyridine hydrochloride†	1 : 2000 +	1 : 1000 + (*ppt.*)	1 : 2000 +	1 : 1000 + (*ppt.*)
		1 : 1000 —	? —	? —	? —
28	1.1.dinaphthyl-2.2.imine	? +	1 : 1000 +	1 : 1000 + (*ppt.*)	1 : 4000 +
		1 : 2000000 —	? —	? —	? —

* This compound dissolved in water without the addition of acid.

† 1 : 1000 solution strongly acid.

serum; thus 1 : 2000000 sterilised staphylococci in watery medium, but 1 : 1000 failed to kill these organisms in serum. This is the most extreme reduction observed in the case of any substance, being twenty times greater than the reduction effected by serum on mercuric chloride.

So far, therefore, it has not been possible to obtain any fragment of the molecule which equals, or even approximates closely to, diaminoacridine in antiseptic properties.

Acridine Group.

The substances which have been tested are included in Table II. The following general conclusions may be drawn from the results :—

Action of the Amino-Groups.—The introduction of amino-groups enhances the antiseptic potency both for *Staphylococcus aureus* and *B. coli*, *e.g.*, acridine [29] and diaminoacridine [35], dimethylacridine [33] and diaminodimethylacridine [48].

Effectiveness in Serum.—Effectiveness in serum is a characteristic of the compounds with unsubstituted amino-groups and especially of the methochlorides of these bases. The further introduction into the diamino compounds of a phenyl-group attached to the medial carbon atom (in position 9) has, however, a marked effect in diminishing the action in serum ; this is exhibited both in the case of 2·7·diamino-3·6·dimethylacridine [48—51] and 2·amino-3·methylnaphthacridine [58—61]. On the other hand, the methochloride of 9·phenylacridine [32] is more active than that of acridine [30].

Comparison of the Antiseptic Power of the Methochloride and the Hydrochloride of the same Base.—The methochloride (or methosulphate or methonitrate) is never less potent than the hydrochloride in the presence of serum and in some cases the increased effectiveness shown by the methochloride is very remarkable, *e.g.*, 2·7·tetraethyldiaminoacridine [55, 56], 2·7·diamino-3·6·dimethyl-9·phenylacridine [50, 51], 2·amino-3·methylnaphthacridine [58, 59], 2·dimethylaminonaphthacridine [62, 63]. In the case of the simplest member of the amino series, 2·7·diaminoacridine [35, 36],* and where the substituents are directly attached to the outer rings as in 2·7·diamino-3·6·dimethylacridine [48, 49], the hydrochloride and the methochloride are practically equal in antiseptic power. It is noteworthy, however, that when the antiseptic power of diaminoacridine is diminished by substitution of ethyl radicals in the amino-groups, the enhanced action of the methochloride [56] over the hydrochloride [55] again becomes apparent. So far, no rational explanation of the enhanced efficacy of the methochloride has suggested itself.†

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* This result has been obtained with carefully purified specimens of these compounds.

† The observations of Crum Brown and Fraser on the change produced in the pharmacological action of alkaloids when a methyl group is attached to a nitrogen

The hydrochlorides of certain of the compounds require the presence of a slight excess of hydrochloric acid in order to effect solution, *e.g.*, in the case of 9.phenylacridine [31] and 2.amino-3.methylnaphthacridine [58]; but the enhanced effect of the methochlorides [32, 59] over the respective hydrochlorides [31, 58] is not to be ascribed to the higher hydrogen-ion concentration of the solution of the latter, since the addition of hydrochloric acid to the methochloride, so as to produce a solution of similar reaction, did not reduce the antiseptic power to that of the hydrochloride. The comparative effects of the hydrochloride and the methochloride of the same base in peptone water show a much less regular behaviour.

The Substitution of other Radicals for the Methyl Group in Diaminoacridine Methochloride.—The following were examined: ethyl [37], propyl [38], *n*- [39] and iso-butyl [40], iso-amyl [41], phenyl [42], benzyl [43], also the chloroacetate [44], chloropropionate [45], and chloroacetanilide [46] derivatives. The result was that, within the limits of experimental variation, these compounds are practically identical with the methochloride [36] in their antiseptic power for both organisms.

The Substitution of Alkyls in the Amino Groups.—Tetramethyl- [52] and tetraethyl-diaminoacridine [55] hydrochloride and also the methochlorides [53, 56] and methonitrate [54, 57] were investigated. The tetramethyl hydrochloride [52], while practically equal to unsubstituted diaminoacridine [35] in its action on *Staphylococcus aureus,* was distinctly inferior for *B. coli,* both in peptone water and in serum. The tetraethyl compound [55] was still weaker; thus with the latter the sterilising concentration for staphylococcus in peptone water was 1 : 100000 and in serum 1 : 10000, while for *B. coli* a concentration not less than 1 : 1000 was required. The methochloride [53] and methonitrate [54] of the tetramethyl compound were practically equal to the hydrochloride; also, as in the case of the unsubstituted diaminoacridine, the effect in serum with the hydrochloride and methochloride was practically equal. On the other hand, the methochloride [56] and methonitrate [57] of the tetraethyl compound were much more active in serum than the hydrochloride.

Groups which interfere with Antiseptic Action.—As has been shown above, the introduction of methyl and ethyl groups into the amino radicals depresses rather than enhances the antiseptic potency [52, 55, as compared with 35], thus contrasting with the effect of similar substituents in the diamino- and

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atom, thus converting the compound into a quaternary base, should be recalled in this connection ('Trans. Roy. Soc.,' Edinburgh, vol. 25, pp. 151, 693 (1868–69); 'Proc. Roy. Soc.,' Edinburgh, vol. 6, p. 556 (1868–69)). It has been shown, however, by Lenz that diaminoacridine methochloride is totally devoid of curare action, either in cold or warm blooded animals ('Zeitschr. f. d. gesamt. experim. Med.,' vol. 12, p. 195 (1921)).

Table II.—Antiseptic Action of Acridine Derivatives.

No.	Substance.	Organism.			
		Staphylococcus aureus : medium		*B. coli* : medium	
		Peptone water.	Serum.	Peptone water.	Serum.
29	Acridine hydrochloride	1 : 20000 + 1 : 2000 –	1 : 4000 + ? –	1 : 2000 + 1 : 1000 –	1 : 1000 + ? –
30	Acridine methochloride	1 : 10000 + 1 : 1000 –	1 : 10000 + 1 : 2000 –	1 : 10000 + 1 : 1000 –	1 : 20000 + 1 : 4000 –
31	9.phenylacridine hydrochloride	1 : 2000 + 1 : 1000 –	1 : 1000 + ? –	1 : 2000 + 1 : 1000 –	1 : 1000 + ? –
32	9.phenylacridine methochloride	1 : 1000000 + 1 : 40000 –	1 : 200000 + 1 : 20000 –	1 : 4000 + 1 : 2000 –	1 : 20000 + 1 : 10000 –
33	3.6.dimethylacridine hydrochloride*	1 : 1000 + (*ppt.*) ? –	1 : 1000 + (*ppt.*) ? –	1 : 1000 + (*ppt.*) ? –	1 : 1000 + (*ppt.*) ? –
34	3.6.dimethylacridine methochloride	1 : 40000 (*inh.*) 1 : 20000 –	1 : 40000 + 1 : 4000 –	1 : 10000 + 1 : 4000 –	1 : 10000 + 1 : 4000 –
35	2.7.diaminoacridine hydrochloride (and sulphate)	1 : 400000 + 1 : 200000 –	1 : 400000 + 1 : 200000 –	1 : 40000 + 1 : 20000 –	1 : 200000 + 1 : 100000 –
36	2.7.diaminoacridine methochloride	1 : 400000 + 1 : 200000 –	1 : 400000 + 1 : 200000 –	1 : 40000 + 1 : 20000 –	1 : 200000 + 1 : 100000 –
37	2.7.diaminoacridine ethochloride	1 : 200000 + 1 : 40000 –	1 : 400000 + 1 : 200000 –	1 : 100000 + 1 : 10000 –	1 : 400000 + 1 : 100000 –
38	2.7.diaminoacridine propylchloride	1 : 100000 + 1 : 40000 –	1 : 400000 + 1 : 200000 –	1 : 40000 + 1 : 10000 –	1 : 400000 + 1 : 200000 –
39	2.7.diaminoacridine *n*-butylchloride	1 : 100000 + 1 : 40000 –	1 : 400000 + 1 : 200000 –	1 : 40000 + 1 : 10000 –	1 : 400000 + 1 : 200000 –
40	2.7.diaminoacridine iso-butylchloride	1 : 20000 + 1 : 10000 –	1 : 400000 + 1 : 200000 –	1 : 100000 + 1 : 40000 –	1 : 400000 + 1 : 200000 –
41	2.7.diaminoacridine iso-amylchloride	1 : 400000 + 1 : 100000 –	1 : 400000 + 1 : 200000 –	1 : 100000 + 1 : 10000 –	1 : 400000 + 1 : 200000 –
42	2.7.diaminoacridine phenylchloride	1 : 200000 + 1 : 40000 –	1 : 400000 + 1 : 100000 –	1 : 100000 + 1 : 10000 –	1 : 400000 + 1 : 100000 –
43	2.7.diaminoacridine benzylchloride	1 : 100000 + 1 : 40000 –	1 : 400000 + 1 : 100000 –	1 : 100000 + 1 : 20000 –	1 : 400000 + 1 : 200000 –
44	2.7.diaminoacridine chloroacetate	1 : 200000 + 1 : 100000 –	1 : 1000000 + 1 : 200000 –	1 : 40000 + 1 : 20000 –	1 : 1000000 + 1 : 400000 –
45	2.7.diaminoacridine chloropropionate	1 : 400000 + 1 : 200000 –	1 : 1000000 + 1 : 400000 –	1 : 200000 + 1 : 40000 –	1 : 1000000 + 1 : 200000 –
46	2.7.diaminoacridine chloracetanilide	1 : 200000 + 1 : 40000 –	1 : 1000000 + 1 : 200000 –	1 : 20000 + 1 : 10000 –	1 : 400000 + 1 : 100000 –

47	2.7.diaminoacridine methochloride + C_2H_5I	1 : 200000 +	1 : 1000000 +	1 : 100000 +	1 : 200000 +
		1 : 100000 −	1 : 100000 −	1 : 40000 −	1 : 40000 −
48	2.7.diamino-3.6.dimethyl-acridine hydrochloride (acridine yellow)	1 : 400000 +	1 : 400000 +	1 : 1000 +	1 : 200000 +
		1 : 20000 −	1 : 100000 −†	? −	1 : 100000 −
49	2.7.diamino-3.6.dimethyl-acridine methochloride	1 : 1000000 +	1 : 400000 +	1 : 40000 +	1 : 400000 +
		1 : 400000 −	1 : 200000 −	1 : 10000 −	1 : 100000 −
50	2.7.diamino-3.6.dimethyl-9.phenylacridine hydrochloride (benzoflavine)	1 : 400000 +	1 : 20000 +	1 : 1000 +	1 : 1000 +
		1 : 200000 −	1 : 10000 −	? −	? −
51	2.7.diamino-3.6.dimethyl-9.phenylacridine methochloride	1 : 4000000 +	1 : 1000000 +	1 : 4000 +	1 : 40000 +
		1 : 2000000 −	1 : 200000 −	1 : 2000 −	1 : 10000 −
52	2.7.tetramethyldiaminoacridine hydrochloride (acridine orange)	1 : 200000 +	1 : 200000 +	1 : 10000 +	1 : 40000 +
		1 : 100000 −	1 : 100000 −	1 : 4000 −	1 : 20000 −
53	2.7.tetramethyldiaminoacridine methochloride	1 : 1000000 +	1 : 400000 +	1 : 40000 +	1 : 100000 +
		1 : 400000 −	1 : 100000 −	1 : 20000 −	1 : 40000 −
54	2.7.tetramethyldiaminoacridine methonitrate	1 : 1000000 +	1 : 400000 +	1 : 20000 +	1 : 100000 +
		1 : 200000 −	1 : 200000 −	1 : 10000 −	1 : 20000 −
55	2.7.tetraethyldiaminoacridine hydrochloride	1 : 200000 +	1 : 20000 +	1 : 4000 +	1 : 1000 +
		1 : 100000 −	1 : 10000 −	1 : 1000 −	? −
56	2.7.tetraethyldiaminoacridine methochloride	1 : 2000000 +	1 : 1000000 +	1 : 40000 +	1 : 40000 +
		1 : 1000000 −	1 : 400000 −	1 : 10000 −	1 : 20000 −
57	2.7.tetraethyldiaminoacridine methonitrate	1 : 2000000 +	1 : 400000 +	1 : 10000 +	1 : 20000 +
		1 : 400000 −	1 : 200000 −	1 : 4000 −	1 : 10000 −
58	2.amino-3.methylnaphthacridine hydrochloride	1 : 400000 +	1 : 40000 +	1 : 20000 + (*ppt.*)	1 : 2000 + (*ppt.*)
		1 : 200000 −	1 : 2000 −	1 : 10000 − (*ppt.*)	1 : 1000 − (*ppt.*)
59	2.amino-3.methylnaphthacridine methochloride	1 : 10000000 +	1 : 1000000 +	1 : 100000 +	1 : 1000000 +
		1 : 1000000 −	1 : 400000 −	1 : 20000 − (*ppt.*)	1 : 100000 −
60	2.amino-3.methyl-9.phenylnaphthacridine hydrochloride	1 : 2500 +	1 : 1666 + (*ppt.*)	1 : 1666 (*inh.*) (*ppt.*)	1 : 1666 + (*ppt.*)
		1 : 1666 − (*ppt.*)	? −	? −	? −
61	2.amino-3.methyl-9.phenylnaphthacridine methosulphate	? +	1 : 100000 (*inh.*)	1 : 10000 + (*ppt.*)	1 : 20000 +
		1 : 100000 −	1 : 40000 −	1 : 1000 − (*ppt.*)	1 : 4000 −
62	2.dimethylaminonaphthacridine hydrochloride	1 : 40000 +	1 : 1000 + (*ppt.*)	1 : 20000 + (*ppt.*)	1 : 1000 + (*ppt.*)
		1 : 20000 − (*ppt.*)	? −	1 : 2000 − (*ppt.*)	? −
63	2.dimethylaminonaphthacridine methochloride	1 : 1000000 (*inh.*)	1 : 400000 +	1 : 20000 +	1 : 40000 +
		1 : 400000 −	1 : 40000 −	1 : 4000 − (*ppt.*)	1 : 20000 −
64	2.7.diacetyldiaminoacridine chloroacetate	1 : 1000 +	1 : 2000 +	1 : 1000 +	1 : 2000 +
		? −	? −	? −	? −
65	2.7.diamino-9.phenylacridine carboxylic ester	1 : 40000 +	1 : 4000 +	1 : 1000 +	1 : 1000 +
		1 : 4000 −	1 : 1000 (*inh.*)	? −	? −
66	2.7.tetramethyldiamino-9.phenylacridine carboxylic ester	1 : 100000 +	1 : 8000 +	1 : 2000 +	1 : 2000 +
		1 : 8000 −	1 : 2000 −	? −	? −
67	2.7.dihydroxy-3.6.dimethyl acridine sodium salt	1 : 1000 +	1 : 1000 +	1 : 1000 +	1 : 1000 +
		? −	? −	? −	? −
68	2.7.dihydroxy-3.6.dimethyl acridine methochloride	1 : 2000 +	1 : 1000 +	1 : 1000 +	1 : 1000 +
		? −	? −	? −	? −

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* The 1 : 1000 solution reacts acid to litmus paper.

† This result was obtained in repeated tests; but in other experiments, again, there was a wider zone of inhibition with 1 : 20000 −

triaminotriphenylmethane dyes (2). The substitution of one hydrogen atom in each of the amino groups by acetyl radicals practically abolishes the antiseptic action, *e.g.*, the sterilising concentration of 2.7. diaminoacridine chloroacetate [44] for *Staphylococcus aureus* in peptone water was 1 : 100000 and in serum 1 : 200000, and for *B. coli* in peptone water 1 : 20000, and in serum 1 : 400000 ; on the other hand, with the diacetyl derivative [64] a concentration of 1 : 2000 failed in each case to sterilise.

The carboxylic esters of 2.7. diamino-9.phenylacridine [65] and of 2.7. tetramethyldiamino-9.phenylacridine [66] were so weak as to suggest a marked depressing effect of the carboxyl group on the antiseptic property.

The replacement of the amino groups by hydroxyls also led to practical abolition of antiseptic power, as is shown in the case of 2.7.dihydroxy-3.6.dimethylacridine, of which both the sodium salt [67] and the methochloride [68] were tested.

Comparative Efficiency for Staphylococcus aureus *and* B. coli.—Antiseptic potency for *Staphylococcus aureus* and *B. coli* does not invariably run parallel ; thus the lethal concentration in serum for staphylococcus is 1 : 100000, or lower in the case of 2.7.diaminoacridine hydrochloride (or sulphate) [35] and methochloride [36], and other analogous derivatives [37–46], 2.7.tetramethyldiaminoacridine hydrochloride [52], methochloride [53] and methonitrate [54], 2.7.diamino- 3.6.dimethyl acridine hydrochloride [48], and methochloride [49], 2.7.tetraethyldiaminoacridine methochloride [56] and methonitrate [57], 2.7.diamino 3.6.dimethyl-9.phenylacridine methochloride [51], 2.amino-3.methylnaphthacridine methochloride [59]. But in the case of *B. coli* only the hydrochloride, methochloride, and analogous derivatives of diaminoacridine [35–46] and of diaminodimethylacridine [48, 49], and the methochloride of 2.amino-3. methyl naphthacridine [59] reach this level of effectiveness.

Phenazine Series.

The striking feature of this series (see Table III) is the relatively poor antiseptic power exhibited by the amino compounds in serum, especially for *B. coli.* The only compounds exactly comparable with the acridine series are those of the phenazine base [69, 70], 2.7.tetramethyl diaminophenazine [80, 81], and 2.7.diamino-3.6.dimethylphenazine [86]. The enhanced effect of the metho-compounds as compared with the hydrochlorides of the same base is evident in the phenazine series ; but is not so striking as with certain of the diaminoacridine derivatives.

The relatively greater efficiency of the methochloride of 2.dimethylamino-7.amino-6.methylphenazine [84], as compared with 2.dimethylamino-7.aminophenazine [76], and of 2.7.diamino-3.6.dimethylphenazine [86] as

Table III.—Antiseptic Action of Phenazine Series in Serum.

No.	Substance.	Organism.			
		Staphylococcus aureus : medium		*B. coli* : medium	
		Peptone water.	Serum.	Peptone water.	Serum.
69	Phenazine hydrochloride		1 : 5000 + ? —		1 : 2500 + ? —
70	Phenazine methochloride		1 : 10000 + 1 : 4000 —		1 : 40000 + 1 : 10000 —
71	Phenazine methosulphate		1 : 10000 + 1 : 4000 —		1 : 40000 + 1 : 10000 —
72	2.aminophenazine hydrochloride		1 : 2000 + ? —		1 : 1000 + ? —
73	2.aminophenazine methochloride		1 : 10000 + 1 : 4000 —		1 : 10000 + 1 : 4000 —
74	2.3.diaminophenazine hydrochloride		1 : 1000 + ? —		1 : 1000 + ? —
75	2.3.diaminophenazine methochloride		1 : 4000 + 1 : 2000 —		1 : 1000 + (*ppt.*) ? —
76	2.dimethylamino-7.aminophenazine methochloride		1 : 100000 + 1 : 2000 —		1 : 1000 + ? —
77	2.dimethylamino-7.amino-6.methylphenazine hydrochloride (see methochloride No. 84)		1 : 1000 + ? —		1 : 1000 + ? —
78	2.dimethylamino-6.methylphenazine hydrochloride		1 : 1000 + ? —		1 : 1000 + ? —
79	2.dimethylamino-6.methylphenazine methiodide		1 : 40000 + 1 : 10000 —		1 : 10000 + 1 : 2000 —
80	2.7.tetramethyldiaminophenazine hydrochloride		1 : 10000* + ? —		1 : 10000* + ? —
81	2.7.tetramethyldiaminophenazine hydriodide		1 : 5000* + ? —		1 : 5000* + ? —

The values obtained with compounds 69–83 in peptone water are omitted on account of insufficient control of the reaction.

* On account of the insolubility of these substances higher concentrations could not be tested.

Table III.—*continued.*

No.	Substance.	Organism.			
		Staphylococcus aureus: medium		*B. coli*: medium	
		Peptone water.	Serum.	Peptone water.	Serum.
82	2.aminonaphthophenazine hydrochloride†		1 : 1000 + (*ppt.*) ? —		1 : 1000 + (*ppt.*) ? —
83	2. aminonaphthophenazine methochloride		1 : 40000 + 1 : 4000 —		1 : 40000 + 1 : 20000 —
84	2.dimethylamino-7.amino-6.methylphenazine methochloride‡	1 : 400000 + 1 : 100000 —	1 : 400000 + 1 : 200000 —	1 : 1000 + ? —	1 : 100000 + 1 : 20000 —
85	2.7.diamino-6.methylphenazine methochloride	1 : 100000 + 1 : 20000 —	1 : 100000 + 1 : 20000 —	1 : 4000 + (*ppt.*) 1 : 1000 — (*ppt.*)	1 : 20000 + 1 : 4000 —
86	2.7.diamino-3.6.dimethylphenazine methochloride	1 : 1000000 (*inh.*) 1 : 200000 —	1 : 400000 (*inh.*) 1 : 100000 —	1 : 1000 + (*ppt.*) ? —	1 : 200000 + 1 : 20000 —
87	2.aminonaphtho-7.amino-6.methylphenazine methochloride	1 : 1000000 (*inh.*) 1 : 400000 —	1 : 400000 (*inh.*) 1 : 200000 —	1 : 20000 + (*ppt.*) ? —	1 : 100000 + 1 : 40000 —
88	2.methylamino-7.amino-3.6.dimethylphenazine methochloride	1 : 1000000 + 1 : 200000 —	1 : 400000 + 1 : 40000 —	1 : 2000 + 1 : 1000 —	1 : 100000 + 1 : 20000 —
89	2.dimethylamino-7.amino-3.6.dimethylphenazine methochloride (zinc chloride compound)	1 : 400000 + 1 : 40000 —	1 : 100000 + 1 : 40000 —	1 : 2000 + ? —	1 : 40000 + 1 : 20000 —
90	2.benzylamino-7.amino-3.6.dimethylphenazine methochloride (zinc chloride compound)	1 : 1000000 + 1 : 400000 —	1 : 40000 + 1 : 10000 —	1 : 10000 + 1 : 2000 —	1 : 200000 + 1 : 4000 —
91	7.amino-6 methyl-2.dimethylamino - naphthophenazine hydrochloride	1 : 40000 + 1 : 10000 —	1 : 10000 + 1 : 1000 — (*ppt.*)	1 : 1000 + (*ppt.*) ? —	1 : 40000 + ? —
92	7.amino-6.methyl-2.dimethylamino - naphthophenazine methochloride	1 : 400000 + 1 : 200000 —	1 : 40000 + 1 : 4000 —	1 : 10000 + 1 : 4000 — (*ppt.*)	1 : 10000 + 1 : 4000 —
93	N-Methyltetrahydroquinoline-2.aminophenazine methochloride (zinc chloride compound)	1 : 400000 + 1 : 100000 —	1 : 200000 + 1 : 20000 —	1 : 4000 + 1 : 2000 —	1 : 100000 + 1 : 20000 —

* On account of the insolubility of these substances higher concentrations could not be tested.

† Only partially dissolved.

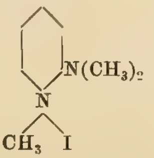
‡ The zinc chloride compound of this substance gave practically the same values.

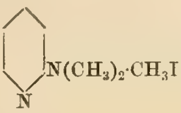
compared with 2.7.diamino-6.methylphenazine [85], suggests that methyl groups attached directly to the benzene rings may play a part in enhancing the antiseptic power in this series. For *Staphylococcus aureus* in serum the methochlorides of 2.dimethyl amino-7.amino-6.methylphenazine [84], 2.7.diamino-3.6.dimethylphenazine [86], and 2.aminonaphtho-7.amino-6.methylphenazine [87] are powerful antiseptics, practically equal to the most potent of the acridine series; on the other hand, they are markedly inferior to the latter in their action on *B. coli.*

It cannot be said that the behaviour of the phenazine series throws any clear light on the antiseptic properties of the diaminoacridine group.

Chemical Section.

Amino Pyridine Derivatives [1.2.].—*α-Amino pyridine* was obtained by the method of Tschtschibabin and Leide.* It melted at 57–58° as stated by the authors of the process. By heating with a slight excess of acetic anhydride and some fused sodium acetate it was converted into the acetyl derivative which solidified on pouring the product into water. To purify it, it was dissolved in benzene filtered from sodium acetate and the benzene removed by distillation. It melted at 72°. The acetyl derivative was warmed with methyl sulphate when the mixture heated spontaneously and on cooling solidified to a pasty crystalline mass, which was drained on a porous plate. The product, on boiling with conc. hydrochloric acid and evaporating, was not the methochloride as it contained no chlorine, but probably undecomposed methosulphate. When aminopyridine was heated in methyl alcohol solution with 4 molecules of methyl iodide in the water-bath in a sealed tube for 6—7 hours, it was converted into the dimethylamino pyridine methiodide which separated on evaporating the methyl alcohol in brown crystals of the periodide. On dissolving the periodide in water iodine separated and the methiodide passed into the solution, which is colourless. On evaporation under diminished pressure the methiodide separated in fine, colourless needles which turned yellow in the air. On analysis the compound gave 50 per cent. of iodine (calculated for $C_5H_5N(CH_3)_2CH_3I$, I = 48·1 per cent.). The constitution is, therefore, represented by one of the following formulæ:—





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* 'J. S. C. I. Ann. Report,' 1915, p. 863.

[4–11.] *The Aminoquinolines and the Methochlorides.*—The *o, m, p* and *a* aminoquinolines were prepared from the corresponding nitroquinolines by reduction, the latter being obtained from the nitranilines by Knueppel's modification of Skraup's method* in which anhydrous arsenic acid is employed as oxidising agent. The *m*-nitraniline gives rise to two nitroquinolines, the *meta* and *ana* derivatives, which were separated by crystallisation from alcohol in which the *ana*-compound, m.p. 65°, dissolves more readily than the *meta*, m.p. 132-4°. In this way the meta-compound was obtained quite pure; but the ana-compound after repeated crystallisation melted at 48–50°.

The reduction of the *o*- and *p*-nitroquinolines was effected by a modification of the process described by Knueppel with iron and hydrochloric acid. The product in the case of the ortho-compound was evaporated to dryness and the residue extracted with alcohol in which the hydrochloride dissolves. The solution was made alkaline and distilled in steam. The distillate was acidified with hydrochloric acid and evaporated to dryness. The base was separated by adding caustic soda and extracting with ether. On removing the ether, the base separated in almost colourless crystals, which after recrystallisation melted at 65°. In the case of the para-compound, the product after reduction was made alkaline and extracted with ether. It melted at 108°. The *m*- and *a*-compounds were reduced with tin and hydrochloric acid. Calculated quantities of the materials were introduced into a flask and heated for a short time in the water-bath until the reaction began when the flask was removed and if necessary cooled. The tin double salt of the meta-compound, which crystallises on cooling, was separated, decomposed with excess of alkali and extracted with ether. The product, crystallised from alcohol, melted at 188–190°. In the case of the ana-compound the double salt does not separate readily and the solution was, therefore, concentrated on the water-bath. The base was extracted with ether after the addition of alkali and on crystallisation from alcohol melted at 107°. The acetyl derivatives were prepared by boiling gently for $\frac{1}{4}$ hour 1 grm. of the base with 1 grm. of fused sodium acetate and 4 c.c. of acetic anhydride. After cooling, water was added and then ammonia gradually until alkaline when the acetyl derivatives crystallised. They were recrystallised from dilute alcohol or water from which the *m*-, *p*- and *a*-compounds separated in colourless plates or flattened prisms; the *o*-compound had a faint yellow colour. The following are the melting points:—

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* 'Ber.,' vol. 29, p. 703 (1896); 'Annalen,' vol. 310, p. 75 (1900).

o-Acetyl aminoquinoline	103°
m- „ „	161°
p- „ „	138°
a- „ „	178°

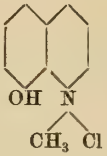
By boiling each of the above with a little conc. hydrochloric acid and concentrating the solution, the pure hydrochlorides crystallised with a brown or yellow colour and were filtered and washed with alcohol.

The hydrochloride of the *m*-compound dissolves in water or alcohol with a bright green fluorescence. All the hydrochlorides combine with potassium cyanate and form orange, crystalline carbamido derivatives.

The methochlorides were obtained from each of the acetyl derivatives by dissolving the substance in three to four times its weight of freshly distilled nitrobenzene and heating the solution to 150°. Rather more than the calculated quantity of dimethyl sulphate was then added and after a minute the mixture was removed from the bath. A portion of the methosulphate crystallised and the remainder was precipitated by adding ether. After standing for a time the nitrobenzene was removed as far as possible by washing with ether by decantation and then evaporating the ether by a current of air. A few cubic centimetres of conc. hydrochloric acid were added and the mixture boiled for $\frac{1}{4}$ hour. The solution was concentrated on the water-bath. The *o*- and *a*-compounds separated on cooling in brown crystalline crusts, the *m*- and *p*-compounds were crystallised from the hydrochloric acid solution by the addition of alcohol and separated in bright yellow needles. The methochloride of the meta-compound dissolves in water with a green fluorescence like the hydrochloride. The other three hydrochlorides and methochlorides exhibit no fluorescence.

[14.13] 8. *Hydroxyquinoline methopicrate and methochloride.*—These substances were prepared from the sulphate of the base (Chinosol) as follows:—

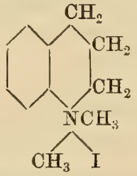
The base was separated from a solution of the sulphate by adding ammonia, filtering and washing with water. It was then dried and heated for an hour with one part of fused sodium acetate and four parts of acetic anhydride. On adding water and a little ammonia until alkaline, the acetyl derivative crystallised in long colourless needles, m.p. 75–7° after recrystallisation from dilute alcohol. The substance was then dissolved in a little toluene and an equal weight of methyl sulphate and boiled for an hour when the yellow crystalline methosulphate separated. The toluene was decanted and the dry residue boiled with conc. hydrochloric acid. In this way a reddish solution was obtained from which the methochloride did not crystallise nor was the base precipitated by ammonia. One portion was, therefore, evaporated to dryness and formed a greenish-yellow solid which is very soluble in water and could not be obtained in the crystalline form from other solvents. To a second portion picric acid was added which precipitated the picrate in yellow clusters of microscopic needles.



8. Hydroxyquinoline methochloride.

[15.] *Tetrahydroquinoline* was prepared according to the method of Hoffmann and Königs,* by reduction with tin and hydrochloric acid. The hydrochloride crystallised from alcohol in colourless needles, m.p. 181–2°.

[16.] *Methyl tetrahydroquinoline methiodide.*—Tetrahydroquinoline obtained from the hydrochloride (2 grm.) was mixed with 14 grm. of methyl iodide at the ordinary temperature. A clear pale orange solution was obtained, from which oily drops separated and gradually solidified to a crystalline mass. The product was warmed on the water-bath with reflux for about 1 hour, and left overnight at room temperature. The excess of methyl iodide was then driven off and the yellowish crystalline mass dissolved in water and filtered. To the filtrate ammonia was added until alkaline and the methyl tetrahydroquinoline extracted with ether. The aqueous solution was evaporated nearly to dryness on the water-bath and cooled. The solid residue was pressed on a porous plate and then extracted with alcohol. On the careful addition of ether the methiodide was precipitated in colourless needles, m.p. 171–2°.



Methyl tetrahydroquinoline methiodide.

[17.] *α-Naphthoquinoline* was prepared by Knueppel's method,† applied to α-naphthylamine by Claus and Imhoff.‡ It was purified by crystallisation from petroleum ether and formed colourless needles, m.p. 45°. The methosulphate was prepared by dissolving the naphthoquinoline in benzene and adding an equal quantity of methyl sulphate and heating on the water-bath for a short time. Pale yellow needles of the quaternary compound separated which were filtered and dried.

The methochloride was obtained by adding conc. hydrochloric acid and boiling for a short time; on adding alcohol and cooling the compound crystallised in colourless needles

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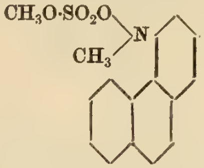
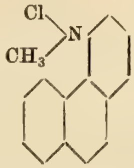
* 'Ber.,' vol. 16, p. 728 (1883).

† 'Ber.,' vol. 29, p. 703 (1896).

‡ 'J. prakt. Chem.,' vol. 57, p. 68 (1898).

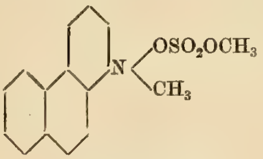


α-Naphthoquinoline methosulphate.

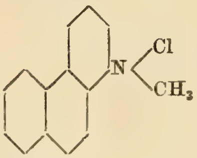


α-Naphthoquinoline methochloride.

[20. 21.] β-Naphthoquinoline was obtained by the method described by Claus and Besseler,* and purified by crystallisation from petroleum ether. It crystallises in pale yellow leaflets, m.p. 93°. It was converted into the methosulphate and chloride (as described under α-naphthoquinoline); *cf.* the following formulæ:—



β-Naphthoquinoline methosulphate.



β-Naphthoquinoline methochloride.

[22.] *Tetrahydro-α-naphthoquinoline* was obtained, as described by Bamberger,† by the reduction of α-naphthoquinoline with tin and hydrochloric acid. The base was precipitated from ethereal solution by hydrogen chloride as a crystalline mass which was purified by re-crystallisation from alcohol acidified with hydrochloric acid. After being decolourised with animal charcoal the hydrochloride of the base was obtained in colourless needles, m.p. 257°–8°.

[23.] *Tetrahydro-β-naphthoquinoline* was prepared from the β-compound, as above, in the form of colourless, lustrous leaflets, m.p. 231°.

[24.] *Diamino-β-naphthoquinoline.*—Five grms. of β-naphthoquinoline, with 6 grms. potassium nitrate and 20 c.c. conc. sulphuric acid, were heated on the water-bath for 3 hours. The nitro-compound was poured on to ice and washed with cold water. It was then warmed on the water-bath with dilute ammonia, which dissolved a brown coloured substance. The product was filtered and dried and crystallised from nitrobenzene. It melted at 230°–245°. On a second crystallisation it melted at 245°–247°. On analysis,

0·16 grm. gave 21·5 c.c. moist N at 18° and 747·5 mm. N = 15·1 per cent.
Calculated for $C_{13}H_7N(NO_2)_2$; N = 15·5 per cent.

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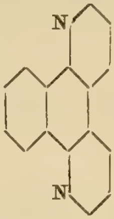
* 'J. prakt. Chem.,' vol. 57, p. 49 (1898).

† 'Ber.,' vol. 24, p. 2475 (1891).

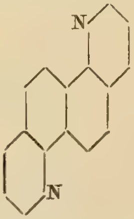
The dinitro-compound was reduced with tin and hydrochloric acid. On cooling the double stannic chloride salt crystallised and was filtered, dissolved in water, and the tin precipitated by hydrogen sulphide. From the filtrate the base was precipitated by sodium hydroxide. It formed pale yellow plates, m.p. 250°. It is very unstable and oxidises in the air.

[26.] 1.4.*Naphtho-dipyridine* (*Benzophenanthroline*) was prepared from 1.4 naphthylene diamine by Karrer's method.* The diamine was obtained by the reduction of benzene-azo-α-naphthylamine with zinc dust and acetic acid; 14·3 grm. of α-naphthylamine gave 21·6 grm. of naphthylenediamine sulphate, from which the base was obtained, m.p. 118°. The naphtho-dipyridine was purified by crystallisation from a mixture of benzene and petroleum ether and separated in long, pale yellow needles, m.p. 160°–164°.



1.4.Naphtho-dipyridine.

[27.] 1.5.*Naphtho-dipyridine.*—The 1.5.naphthylenediamine was prepared by the reduction of 1.5.dinitro-naphthalene with stannous chloride and hydrochloric acid. The naphtho-dipyridine compound was obtained, as previously described, and crystallised from benzene in nearly colourless needles, m.p. 214°–215°. It combines with methyl sulphate in hot benzene solution, from which yellow needles separated on cooling.



1.5.Naphtho-dipyridine.

[28.] 1.1.*Dinaphthyl.*2 2-*imine* was prepared by the method described by Meisenheimer and Witte† from β-naphthylamine. The latter was converted into azonaphthalene which was reduced with zinc dust and acetic acid. In this way 2.2.diamino-1.1.dinaphthyl was obtained, m.p. 191°; 2 grms. of the diamino-dinaphthyl hydrochloride were heated in the oil-bath to 240°–250°

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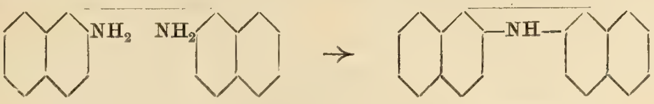
* Marckwald, 'Annalen,' vol. 274, p. 365 (1893).

† 'Ber.,' vol. 36, p. 4154 (1903).

for 5 minutes. The cold fused mass was extracted with alcohol, poured into water, and the colourless precipitate filtered. The yield is nearly theoretical, and the product melted at 157°.



Diamino dinaphthyl. Dinaphthylimine.

Acridine Derivatives: [30] *Acridine Methochloride.*—Acridine base was precipitated from pure acridine hydrochloride by the addition of ammonia, filtered and dried: 1·5 grms. was dissolved in 7 grms. of nitrobenzene, heated to 150°, and 1·7 grms. of methyl sulphate added, and, after 1 minute, cooled. Yellow prisms of the methosulphate separated and were filtered and washed free from nitrobenzene with ether. The methosulphate is soluble in water, and from the solution the methoxide was precipitated with ammonia and crystallised from alcohol in which it is much less soluble than acridine. It crystallises in colourless plates, m.p. 140°. It dissolves readily in hydrochloric acid and the methochloride crystallises on concentration. It may be re-crystallised from alcohol and ether. It is very soluble in water, to which it imparts a green fluorescence.

[32.] 9-*Phenylacridine methochloride.*—9-Phenylacridine was prepared by the method of Bernthsen*. 50 grms. of benzoic acid and 70 grms. of diphenylamine gave 35 grms. of phenylacridine, m.p. 183°. To convert it into the methochloride the above method was adopted. The methosulphate does not separate on cooling, but on adding ether a brown oil was precipitated, which soon solidified. The ether solution was decanted and the methosulphate washed with ether. Concentrated hydrochloric acid was then added and the mixture boiled gently for a short time, when, on cooling, the methochloride separated in green leaflets, which readily dissolve in water with a yellow colour.

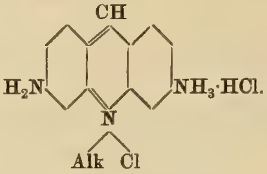
[33.] 3.6.*Dimethylacridine* was prepared according to Ullmann's method.† It crystallises from dilute alcohol in yellow needles, m.p. 171°, which fluoresce in solution with a blue colour.

The action of ethyl iodide on acriflavine in ethyl alcohol was examined with the object of obtaining the tetraethyl derivative. The mixture heated to 100° for 6 hours in a sealed tube gave a red crystalline product, which appeared to be a periodide. Its antiseptic action was inferior to that of acriflavine and it was not further investigated. It is recorded in the Table as acriflavine $+ C_2H_5I$.

* 'Annalen,' vol. 224, p. 13 (1884).

† 'Ber.,' vol. 36, p. 1017 (1903).

[36–43.] 2.7.*Diaminoacridine Alkyl Chlorides.*—In order to study the relative antiseptic action of the quaternary alkyl derivatives of diaminoacridine in addition to the methochloride (acriflavine), the ethyl, propyl, *n*-butyl, isobutyl, isoamyl, phenyl, and benzyl chlorides were prepared, having the following general formula:—



The alkyl chlorides were obtained by the following method: A weighed quantity of sodium was dissolved in the alcohol corresponding to the required alkyl compound, and the theoretical amount of *p*-toluene sulphonic chloride added. After standing for a time, the product was shaken two or three times with water. The washed product was dissolved in ether, dried over calcium chloride, the ether distilled, and the residue heated *in vacuo* on the water-bath. In this way the alkyl *p*-toluene sulphonic esters were obtained in the form of pale yellow oils.

$$CH_3{\cdot}C_6H_4{\cdot}SO_2Cl + RONa = CH_3{\cdot}C_6H_4{\cdot}SO_3R + NaCl.$$

About 1 grm. of each ester was allowed to react with an equivalent weight of diacetyldiaminoacridine in nitrobenzene solution at 150°–160° The product in each case was hydrolysed with conc. hydrochloric acid, whereby the alkyl chloride was obtained.

The benzyl chloride compound, which was prepared in a similar way, was a reddish brown crystalline substance, and on analysis

0·152 grm. gave 0·132 grm. AgCl. $Cl = 21{\cdot}6$ per cent. Calculated for $C_{20}H_{17}N_3Cl_2$; $Cl = 19{\cdot}2$ per cent.

The phenyl chloride compound was obtained by dissolving phenol in the calculated quantity of sodium hydroxide solution to form sodium phenate. The equivalent amount of *p*-toluene sulphonic chloride was then added and the mixture boiled for about half an hour, and cooled. The phenyl *p*-toluene sulphonic ester which separated was recrystallised from alcohol and melted at 95°. It was transformed into the acridine phenochloride in the following way: About 10 per cent. more than the theoretical amount of the ester was added to the acetyl diaminoacridine dissolved in nitrobenzene and heated for 5 minutes to 150°–160°. The product was cooled to 105°, when 5 c.c. of conc. hydrochloric acid was added and the mixture maintained at 100° for another 5 minutes and cooled to the ordinary temperature. The nitrobenzene was decanted from the phenochloride, which separated, and the latter boiled with a little more conc. hydrochloric acid, cooled, and the crystalline product filtered, washed with conc. hydrochloric acid, and finally with ether.

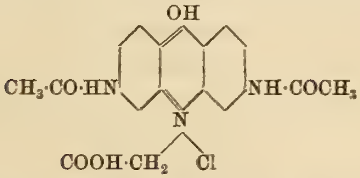
[44.] 2.7.*Diaminoacridine Chloroacetate.*—About 1 grm. of diacetyl diaminoacridine was dissolved in about 50 c.c. of nitrobenzene and heated in a metal bath to 140°–150°. An equivalent amount of chloracetic acid was added and maintained at 140°–150° for about 10 minutes. On cooling, yellow crystals separated, which were filtered and washed with ether. The substance was readily soluble in water. On analysis,

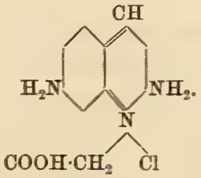
0·1485 grm. gave 0·055 grm. AgCl; Cl = 9·0 per cent. Calculated for $C_{19}H_{17}N_3O_4Cl$; Cl = 9·05 per cent.

On boiling the diacetyl derivative with conc. hydrochloric acid, red crystals were obtained, which were purified by dissolving in water and reprecipitating with conc. hydrochloric acid.

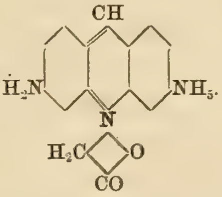
On analysis:

0·141 grm. gave 0·127 grm. AgCl; Cl = 22·2 per cent. Calculated for $C_{15}H_{13}N_3O_2Cl_2$; Cl = 20·9 per cent.





On addition of ammonia it was converted into the betaïne

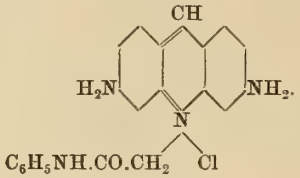


[46.] 2.7.*Diaminoacridine Chloracetanilide* was prepared by adding an equivalent of chloracetanilide to diacetyl diaminoacridine in nitrobenzene (1 grm. of the diacetyl derivative in 50° c.c. of nitrobenzene), heated to 140°–150° and maintained for 5 to 10 minutes. On cooling, an amorphous brown precipitate separated, which was filtered, washed with ether, and boiled with conc. hydrochloric acid. Brown crystals were obtained and were purified by dissolving them in water, and reprecipitating with conc. hydrochloric acid.

On analysis:

0·130 grm. gave 14·5 c.c. N at 15° and 757 mm. N = 13 per cent.
Calculated for $C_{21}H_{19}N_4OCl_2$; N = 13·4 per cent.

The compound has therefore the following formula :—



[45.] 2·7.*Diaminoacridine Chloropropionate* was prepared like the chloracetate and was purified by crystallisation from concentrated hydrochloric acid.

[48.] 2.7.*Diamino*-3.6.*dimethylacridine* (Acridine yellow) was prepared according to the method of Ullmann and Naef[*] and Ullmann and Marie.[†] To purify it, it was ground while moist and warmed on the water-bath with sufficient sodium hydroxide to render the mixture alkaline; after cooling it was filtered, washed and pressed down. The moist precipitate was then dissolved in a small quantity of glacial acetic acid and whilst hot a little conc. hydrochloric added gradually until the solution changed to a deep red, when water was added till turbid and cooled. The hydrochloride separates in fine prismatic, deep orange or red crystals which dissolve in water with a green fluorescence.

[49.] 2.7.*Diamino*-3.6.*dimethylacridine methochloride.*—The hydrochloride prepared as above was precipitated as the base with sodium hydroxide, filtered, washed and thoroughly dried in a vacuum desiccator and then converted into the diacetyl derivative. 3·6 grms. of the base were mixed with 13 grms. of acetic anhydride in a small flask furnished with an air condenser and boiled gently for $\frac{1}{4}$ hour. The substance gradually dissolved. It was diluted with water, and to the cooled solution just sufficient ammonia was added to decompose the anhydride and precipitate the acetyl derivative. After filtering, washing and drying it was extracted with a little absolute alcohol, which removes a small quantity of a pale yellow substance. 1 grm. of the acetyl derivative was dissolved in 10 grms. of nitrobenzene, heated to 170° and 0·8 grm. of methyl sulphate added. On cooling, the mixture solidifies to a crystalline mass which was filtered and washed free from nitrobenzene with ether. The crystalline residue was boiled with conc. hydrochloric acid for $\frac{1}{2}$ hour, when nearly the whole dissolved with a deep red colour. It was filtered and cooled. The methochloride separates in brown, glistening plates, which dissolve in water but are less soluble in dilute hydrochloric acid. It dissolves in alcohol with green fluorescence.

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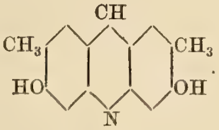
[*] 'Ber.,' vol. 33, p. 913 (1900).

[†] 'Ber.,' vol. 34, p. 4308 (1908).

[67.] 2.7.*Dihydroxy*-3.6.*dimethyl acridine.*—The compound was obtained from tetramethyl diamino ditolylmethane, by the method of Ullmann and Fitzenkam*



It was converted into the diacetyl derivative by boiling with acetic anhydride and fused sodium acetate.

[68.] 2.7.*Dihydroxy*-3.6.*dimethyl acridine methochloride.*—The acetyl derivative was dissolved in toluene and to the solution an excess of methyl sulphate was added and the mixture boiled. On standing, orange needles separated and were filtered and washed with toluene. On heating the substance with conc. hydrochloric acid on the water-bath, it first dissolved and then suddenly formed a pasty mass of clusters of lemon yellow needles of the methochloride. The crystals were filtered and washed with cold water and then with alcohol. In the latter it dissolves slightly with green fluorescence. It dissolves to some extent also in hot water with a yellow colour, and is readily soluble in ammonia and sodium hydroxide solutions.

[50.] 2.7.*Diamino*-3.6.*dimethyl*-9.*phenyl acridine* (Benzoflavine) was prepared according to the method of Meyer and Gross.† It was purified by precipitating the base with ammonia, dissolving the filtered, washed and well-pressed base in glacial acetic acid, adding conc. hydrochloric acid and diluting until a permanent turbidity was formed. The hydrochloride separates in red crystals.

[51.] 2.7.*Diamino*-3.6.*dimethyl*.9-*phenyl acridine methochloride* was obtained by the method employed in the case of acridine yellow by heating the dry base with acetic anhydride and methylating the acetyl derivative with methyl sulphate. On hydrolysis of the methosulphate with hydrochloric acid the methochloride separates in bright red needles. In the absence of hydrochloric acid, it dissolves in water and on cooling gelatinises.

[52.] 2.7.*Tetramethyl diamino-acridine* (Acridine orange) was obtained by the method of Behringer‡ and Ullmann and Marie.§ Tetramethyl-diamino diphenylmethane, prepared by the action of formaldehyde on dimethyl aniline in presence of dilute sulphuric acid was nitrated in the cold with potassium nitrate and conc. sulphuric acid and the purified dinitro-derivate, m.p. 141°–2°, reduced with stannous chloride. After reduction the base m.p. 141°–2° was precipitated with caustic soda and extracted from the stannous hydroxide with alcohol. It was converted into the acridine compound m.p. 181°–2° as described under tetraethyldiamino acridine (see below) and the latter into the methosulphate. From the solution of the methosulphate potassium nitrate precipitates the methonitrate in red needles from which the methoxide was obtained as a hygroscopic mass on the addition of ammonia. The methochloride is too soluble and hygroscopic to be separated, and closely resembles the corresponding ethyl derivative described below.

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* 'Ber.,' vol. 38, p. 3787 (1905).

† 'Ber.,' vol. 32, p. 2356 (1899).

‡ 'J. prakt. Chem.,' vol. 54, p. 240 (1896).

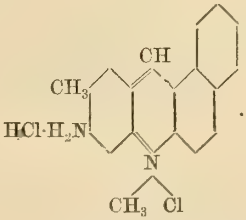
§ 'Ber.,' vol. 34, p. 4307 (1901).

[55.] 2.7.*Tetraethyldiamino-acridine* was prepared by the above method using diethylaniline in place of dimethylaniline. The tetraethyldiamino diphenyl methane is a colourless liquid, which boils at 285°–290° at 10 mm. and 305°–310° at 20 mm. pressure. The yield from 100 grms. of diethylaniline was 50 grm. of the diphenylmethane derivative. On nitration it formed a dinitro derivative which crystallised from acetic acid in orange red plates m.p. 118°–119°. The latter was reduced with tin and hydrochloric acid and the tin partly removed by hydrogen sulphide. The filtered solution on evaporation left a colourless, resinous mass of the hydrochloride which contained some tin salt. 2 grms. of the hydrochloride were heated with 4 c.c. of conc. hydrochloric acid diluted with 12 c.c. of water at 140° for 6 hours in a sealed tube. The red coloured contents of the tube were extracted with boiling water in which they dissolved leaving very little residue, and to the hot solution ferric chloride solution was added until no more precipitate was formed. After the addition of a solution of common salt, which throws down a further quantity of the acridine compound, the mixture was filtered after cooling and washed with salt solution. The deep red solution was made alkaline with ammonia which precipitates the yellow base. The latter was filtered and washed and dissolved in hydrochloric acid. On concentrating the solution, the hydrochloride crystallises in red crusts with a green iridescence. The crystals dissolve in water with a bright orange colour.

[56. 57.] 2.7.*Tetraethyldiamino-acridine methochloride and methonitrate.*—To prepare the methonitrate an excess of dimethyl sulphate was added to the finely powdered base in a small basin. The mixture becomes hot. After heating for a time on the water-bath, the methosulphate crystallises. Water was then added, and heating continued until the excess of methyl sulphate was decomposed and a clear red solution obtained. On the addition of a saturated solution of potassium nitrate, the methonitrate crystallised on standing in red needles. They were filtered, washed with a little water, and dried. On grinding with ammonia solution the methoxide was obtained as a sticky red mass with green iridescence which dissolves in hydrochloric acid forming the methochloride as a hygroscopic mass which could not be prepared in the crystalline state.

[58.] 2.*Amino* - 3.*methylnaphthacridine* was prepared according to the directions of Ullmann and Naef.* The base, after re-crystallisation from xylene, melts at 244°. It was acetylated, as described, and the acetyl derivative melted at 320°–321°.

[59.] 2.*Amino* - 3.*methylnaphthacridine methochloride.*—0·6 grm. of the acetyl derivative was dissolved in 2 c.c. of nitrobenzene heated to 160°, and 0·3 grm. of dimethyl sulphate added when the mixture crystallised to a semi-solid mass, which was filtered and washed with ether. The product, after boiling with conc. hydrochloric acid, became dark red, and on concentrating the solution on the water-bath and cooling the methochloride crystallised in long red needles.



[60.] 2.*Amino* - 3.*methyl* - 9.*phenylnaphthacridine.* — The substance was prepared by adding 5 grms. of tetramino-ditolylphenyl methane (Meyer and Gross),† to 8 grms. of β-naphthol at 150° and heating for an hour to 180°–200°. The sticky product was dissolved in 10 c.c. of glacial acetic acid, diluted with an equal volume of water, and poured into 10 grm. of caustic soda in 50 c.c. of water. The yellow precipitate was filtered and washed with dilute caustic soda solution and hot water to remove the naphthol, and the product crystallised from alcohol. The undissolved portion was filtered off. It crystallises from nitrobenzene and also from benzene in yellowish brown needles, m.p. 264°–265°. The alcoholic filtrate was heated to boiling, water added till turbid, and allowed to cool. The crystalline product was re-crystallised from benzene from which it separates in yellow needles, m.p. 269°–271°.

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* 'Ber.,' vol. 33, pp. 915, 2473 (1900).

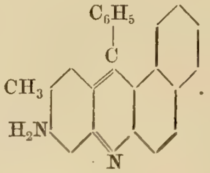
† 'Ber.,' vol. 32, p. 2356 (1899).

The compound of m.p. 264°–265° on analysis

0·2026 grm. gave 14·3 c.c. moist N at 14° and 725 mm. N = 8·37 per cent.

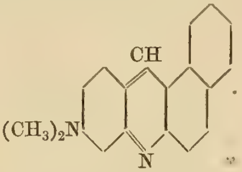
0·1960 grm. gave 13·7 c.c. moist N at 16° and 753 mm. N = 8·24 per cent. Calculated for $C_{24}H_{18}N_2$; N = 8·4 per cent.

The compound has therefore the formula:



It was converted into the methosulphate by dissolving in eight times the weight of nitrobenzene at 130° and adding half the weight of methylsulphate. The crystalline product was filtered and washed with ether.

[62. 63.] 2.*Dimethylaminonaphthacridine and methochloride.*—



It was prepared by the method of Ullmann and Marie,* by fusing tetramethyldiaminodiphenyl methane with β-naphthol at 110°–120°, and raising the temperature gradually to 180°–200°. The product was extracted with warm alcohol which dissolves the naphthacridine compound, but leaves the hydracridine. The hydracridine compound was suspended in boiling alcohol, acidified with a few drops of hydrochloric acid and ferric chloride added until the precipitation of the naphthacridine hydrochloride was complete. The precipitate was filtered, dissolved in water, and re-precipitated with conc. hydrochloric acid. It was filtered and dried *in vacuo* over caustic soda. The base was precipitated from the dissolved hydrochloride with ammonia, and after re-crystallisation from benzene melted at 185°. It was dissolved in boiling xylene and the calculated amount of methyl sulphate added. The precipitated methosulphate was washed with ether and dried; on dissolving it in water and adding sodium chloride solution the methochloride was precipitated, filtered, and dried. To remove the sodium chloride the methochloride was dissolved in alcohol, filtered, and evaporated to dryness. The naphthacridine base was obtained from the first alcoholic filtrate by adding a boiling alcoholic solution of picric acid. The picrate which crystallised was filtered and re-crystallised from boiling aniline. To the picrate suspended in alcohol, caustic soda was added and warmed on the water-bath until the solution was deep yellow, and then diluted. The precipitated base was filtered, washed with water, dried and re-crystallised from benzene (m.p. 185°).

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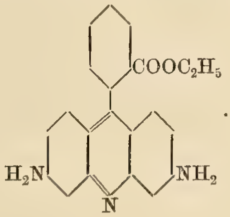
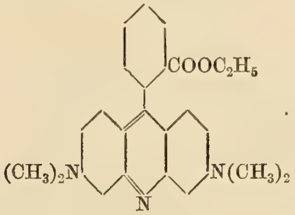
* 'Ber.,' vol. 34, p. 4318 (1901).

[65.] 2.7.*Diamino-9.phenylacridine carboxylic ester.*—



The substance was prepared by heating with ammonia in sealed tubes according to the method of Meyer and Oppelt.* The product was boiled with alcohol to remove impurities and the residue suspended in alcohol and hydrogen chloride passed in. The alcohol was removed, the hydrochloride dissolved in water and precipitated with salt, redissolved and again salted out. The product is an orange amorphous powder.

[66.] 2.7.*Tetramethyldiamino-9.phenylacridine carboxylic ester.*



One molecular proportion of phthalic anhydride was heated with three of acetic anhydride and two of *m*-aminodimethylaniline for 2–3 hours at 140°–150°. The acetic acid was then distilled off and the residue boiled with fifteen to twenty parts of 20 per cent. hydrochloric acid for ½ hour. From the deep red solution the base was precipitated by ammonia, filtered and dried in vacuo. It was suspended in ten times its weight of absolute alcohol, heated an hour with reflux on the water-bath whilst dry hydrogen chloride was passed in. The alcohol was removed, the residue dissolved in hot water, filtered and the filtrate salted out. The precipitate was filtered, washed with a little cold water and dried.

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* 'Ber.,' vol. 21, p. 3376 (1888).

PHENAZINE DERIVATIVES.

[69–71.] Phenazine was prepared by the method of Kehrmann and Havas* by heating a mixture of *o*-amino- and *o*-nitro-diphenylamine with fused sodium acetate. It melts at 170°—171°. The methosulphate and methochloride were obtained in the same manner as the corresponding acridine compounds. The phenazine was dissolved in five times its weight of nitrobenzene, heated to 120°, and freshly distilled methyl sulphate equal in weight to the phenazine was added, the mixture stirred and kept for 5 minutes at 100°–110° and cooled. The methosulphate separates; ether was added to complete the precipitation and the product filtered and washed. It forms greenish yellow prisms. The methochloride was prepared by first separating the base with ammonia and evaporating the red solution, taking up with alcohol to remove ammonium sulphate and then evaporating to dryness. The methochloride was prepared by dissolving the base in hydrochloric acid and concentrating the solution. It forms greenish crystals.

[72.] 2.*Aminophenazine* was prepared by (A) the method described by Wohl and Lange,† from *o*-nitraniline and aniline-hydrochloride in presence of fused zinc chloride. The amino-phenazine was separated from the product by sublimation and crystallises in brilliant red needles melting at 283°, which after crystallisation melt at 288°. The acetyl derivative was obtained by heating for a short time with an equal weight of fused sodium acetate, and twelve to fifteen times its weight of acetic anhydride. When poured into water and neutralised with ammonia it formed a buff-coloured crystalline powder. The dry acetyl derivative was dissolved in ten times its weight of nitrobenzene heated to 120° and methyl sulphate, equal in weight to the acetyl derivative, added and the mixture cooled slowly. Ether precipitates the methosulphate, which was filtered and washed with ether. It was then boiled with water to remove nitrobenzene and filtered. About an equal volume of conc. hydrochloric acid was added and evaporated to dryness. The methochloride, which is very soluble, was dissolved in alcohol, filtered and evaporated. It forms a red, crystalline residue which dissolves in water and alcohol with a bright magenta colour.

(B) A second process for preparing aminophenazine is to pass dry ammonia into an alcoholic solution of phenazine methyl sulphate according to the method of Kehrmann and Havas.‡ The products in the two cases appeared to be identical and to have identical bactericidal properties.

[74.] 2.3.*Diaminophenazine* was prepared by the method of Ullmann and Mauther,* and converted into the acetyl derivative.† It was recrystallised from nitrobenzene and forms a light brown micro-crystalline powder which turns brown at 206° and melts about 270°. The latter was dissolved in ten times its weight of nitrobenzene at 150°, and 1 mol. of methyl sulphate added. The methosulphate, which was precipitated, was washed with ether and the product boiled with conc. hydrochloric acid and evaporated on the water-bath. The methochloride crystallised in black needles which were filtered and dried.

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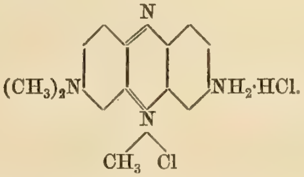
* 'Ber.,' vol. 46, p. 341 (1913).

† 'Ber.,' vol. 43, p. 2186 (1910).

‡ 'Ber.,' vol. 46, p. 431 (1913).

[76.] 2.*Dimethylamino*-7.*aminophenazine methochloride.* — The compound was prepared according to the method described by Karrer,‡ from a mixture of para- and meta-dimethyl phenylene diamine by oxidation with potassium dichromate. The phenazine salt was precipitated by zinc and sodium chloride and the base separated from the solution in hydrochloric acid by sodium hydroxide. It was purified by redissolving in acetic acid and precipitating with ammonia. It forms a blue-black powder slightly soluble in alcohol with a deep violet colour, and readily soluble in dilute acids giving a violet solution and forming the hydrochloride



[77.] 2.*Dimethylamino*-7.*amino*-6.*methylphenazine hydrochloride* (*Toluylene red*).—The substance was obtained by following the directions of Witt.§ It was purified by crystallisation from alcohol. The base was obtained by precipitation with sodium hydroxide, and was washed, dried and acetylated in the usual way. The acetyl derivative crystallises in brown plates. The methiodide was obtained in microscopic black crystals which dissolve in water and alcohol with a deep violet colour, by heating the acetyl derivative with methyl iodide in a sealed tube for several hours to 100°.

[84.] 2.*Dimethylamino*-7-*amino*-6.*methylphenazine methochloride.* — The above acetyl derivative was dissolved in five times its weight of nitrobenzene heated to 160°, and rather more than the calculated weight of methyl sulphate added. After a minute or two the mixture was cooled and the resulting black precipitate washed with ether and dried. The product was then hydrolysed with conc. hydrochloric acid, when the liquid became deep

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[*] 'Ber.,' vol. 35, p. 4302 (1902).

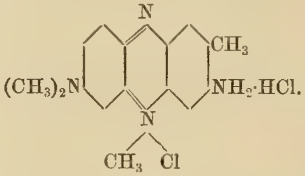
[†] Fischer and Hepp, 'Ber.,' vol. 22, p. 358 (1889).

[‡] 'Ber.,' vol. 49, p. 1643 (1916); vol. 50, p. 420 (1917).

[§] 'Ber.,' vol. 12, p. 921 (1879).

blue. On adding water or salt solution, the methochloride separated in the form of a semi-solid iridescent green mass, which after drying became hard and was crystallised from a mixture of benzene and alcohol in the form of a micro-crystalline powder with a dark green lustre. It dissolves in water and alcohol with a bright magenta colour, similar to toluylene red. On analysis, found $N = 17{\cdot}0$ per cent.; $CH_{20}N_4Cl_2$ requires $N = 16{\cdot}5$ per cent.

The formula is therefore



Another method for the preparation is given in D.R. Patent 69188.* It consists in heating together 3 mols. *p*-nitrosodimethylaniline hydrochloride and 2 mols. *o*-aminodimethyl-*p*-toluidine in 50 per cent. acetic acid solution for 6 hours. On dilution, filtration, and precipitation with common salt, a green crystalline colouring matter was precipitated, which was filtered and, on account of its solubility in water, washed with brine. To the mother liquors, which still contained a quantity of the dye zinc chloride was added, and the zinc chloride double salt precipitated which was re-crystallised from dilute hydrochloric acid. The substance dissolved with a distinctly magenta colour. There appears, therefore, to be a graduation in tint from scarlet to magenta with the increase of alkyl radicals in the amino group (compare [86] and [88]). The same development of blue in the colour is observed in rosaniline and its methyl derivatives which change from magenta to violet.

Bromination of Acetyl Derivative of Toluylene Red.—The acetyl derivative was brominated in chloroform solution, when a dark violet solution was formed, which on evaporation gave a dark violet product, which crystallised from a mixture of alcohol and ether in microscopic black prisms. The substance is, however, a mixture, which can be separated by extracting with ethyl acetate. The undissolved portion was dissolved in water and the base precipitated with ammonia as an oil which solidified on standing. It crystallises from alcohol in pale brown transparent prisms, m.p. 205°–207°. It was tested qualitatively for bromine, but its further investigation was postponed.

[78.] 2.*Dimethylamino*-6.*methylphenazine.*—Toluylene red was diazotised according to Witt's method (*loc. cit.*) in absolute alcohol with sodium nitrite and hydrochloric acid. The excess of alcohol was then removed on the water-bath, the liquid filtered and poured into a solution of sodium acetate, which throws down the phenazine compound in the form of a crystalline precipitate having a bronzy iridescence.

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* 'Friedländer,' vol. 3, p. 397.

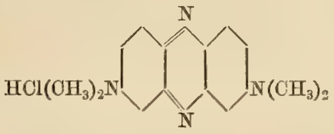
It was recrystallised from dilute alcohol and melted at 168°–169°. The ether and benzene solutions are fluorescent, but fluoresce with different colours, the former having an orange and the latter a greenish colour.

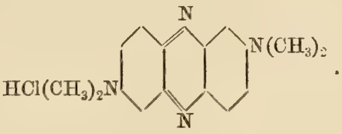
[79.] 2.*Dimethylamino*-6.*methylphenazine methiodide* was prepared from the phenazine compound by heating with rather more than the calculated quantity of methyl iodide in a sealed tube to 100° for 6 hours. A solid dark violet mass resulted, which dissolved in water with a deep violet colour. The substance was recrystallised from a mixture of alcohol and ether, from which it separated in brownish black plates. On analysis,

0·2525 grm. gave 0·1718 grm. AgI; I = 36·8 per cent.
0·2540 grm. gave 0·1714 grm. AgI; I = 37·0 per cent. $C_{15}H_{15}N_3+CH_3I$ requires I = 33·5 per cent.

The iodine value found is too high, the reason for which is not clear unless, as often happens in such cases, a certain amount of periodide is formed at the same time.

[80, 81.] 2.7.*Tetramethyldiaminophenazine* is described by Karrer* as being obtained by oxidising a mixture of dimethyl-*p*-phenylenediamine and dimethyl-*m*-phenylenediamine with potassium dichromate solution in presence of hydrochloric acid. The product may have either of the following formulæ:—





It was separated in the form of the hydriodide, from which the base was precipitated with ammonia, filtered, washed, and extracted with alcohol. The residue was dissolved in dilute hydrochloric acid and the solution was evaporated in a vacuum desiccator to dryness. The methochloride was prepared in the usual way by the action of methyl sulphate on the base, dissolved in nitrobenzene and subsequent hydrolysis with conc. hydrochloric acid. It dissolves in water with a bluish red colour.

[82, 83.] 2. *Aminonaphthophenazine* was prepared, according to the method described by O. Fischer and Hepp,† by heating in a sealed tube for 5 to 6 hours at 160° one molecule *o*-phenylenediamine and one molecule benzene-azo-α-naphthylamine hydrochloride with ten parts of alcohol. On cooling, dark red crystals separated. They were filtered and crystallised from alcohol containing hydrochloric acid. The substance obtained in this way is the hydrochloride of aminonaphthophenazine. It forms dark red crystals, which are slightly soluble in water, but on heating are hydrolysed and the base is precipitated. The acetyl derivative was prepared by heating the base with acetic anhydride and fused sodium acetate. On cooling, the acetyl derivative crystallises. It was heated to 160° with ten to twenty times its weight of nitrobenzene and the equivalent of one molecule of methyl sulphate added. On standing, crystals of the metho-sulphate separated. They were filtered and washed with ether and heated with conc. hydrochloric acid, when the hydrochloride of the methochloride slowly crystallised. The substance is soluble in water and not so readily hydrolysed as the hydrochloride.

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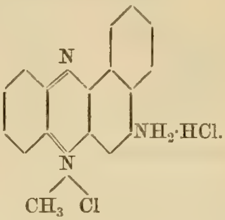
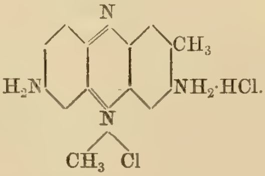
* 'Ber.,' vol. 49, p. 1643 (1916).

† 'Ber.,' vol. 23, p. 845 (1890).



2. Amino-naphtho phenazine methochloride.

[85.] 2.7.*Diamino*-6.*methylphenazine methochloride* was prepared according to D.R. Patent 86608* by heating together and stirring amino-azobenzene hydrochloride, amino dimethyl-*p*-toluidine and glycerol at 110°, according to the proportionate amounts given. The mixture froths up during the process, and the reaction is at an end when frothing ceases (about 3 hours). The solid product was dissolved in hot water and precipitated as hydrochloride by the addition of hydrochloric acid and some common salt. The colouring matter was re-crystallised from dilute hydrochloric acid, giving green, glistening crystals, which dissolved in water with a scarlet colour.



[86.] 2.7.*Diamino*-3.6.*dimethylphenazine methochloride* was prepared as above, using *o*-amino azotoluene hydrochloride in place of the benzene derivative. The product was dissolved in hot water and precipitated with hydrochloric acid. On cooling the phenazine compound separated in green prismatic needles, very slightly soluble in cold water, soluble in hot water with a scarlet colour.

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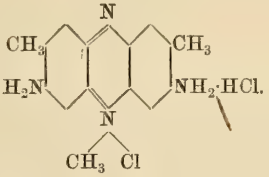
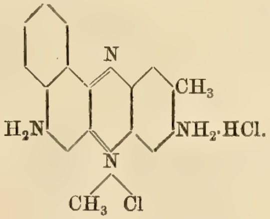
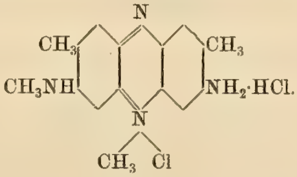
* 'Friedländer,' vol. 4, p. 380.



[87.] 2.*Aminonaphtho*-7.*amino*-6.*methylphenazine methochloride.*—The preparation was carried out as above, using proportionate quantities of benzene-azonaphthylamine hydrochloride and one-and-a-half times the amount of glycerol, and heating at 120° for 3½ hours. The hot aqueous solution of the product was filtered from insoluble matter, and the colouring matter precipitated with hydrochloric acid and salt. It was re-crystallised from dilute hydrochloric acid from which it separated in small green crystals. It dissolves in water with a scarlet colour similar in tint to the other two.



[88.] 2.*Methylamino*-7.*amino*-3.6.*dimethylphenazine methochloride.*—The substance was prepared from nitrosomethylaniline hydrochloride and *o*-amino-*p*-dimethyl toluidine dissolved in alcohol according to the quantities given in D.R. Patent 80758.* The mixture was boiled for 6 hours. The scarlet colour developed in a few minutes and intensified rapidly, until signs of precipitation appeared when the liquid was somewhat concentrated and cooled. The precipitate was filtered and washed with a little alcohol. The phenazine compound consists of a green crystalline powder which dissolves in water with a more magenta colour than the previous compound.



[93.] *N-Methyltetrahydroquinoline-2.aminophenazine methochloride.* — The tetrahydroquinoline and its N-methyl derivative were prepared by the method of Hoffman and Königs,* and converted into the nitroso compound.† A mixture of 2·6 grm. nitroso N-methyl tetrahydroquinoline, 1·5 grm. of *p*-dimethylamino *o*-toluidine, 15 c.c. of glacial acetic acid and 2 c.c. of conc. hydrochloric acid were warmed gently on the water-bath. Heat was developed and the liquid became a brilliant green which rapidly changed through brown black, dull scarlet to magenta. The heating was continued from 3 to 4 hours, when the product was dissolved in 50 c.c. of hot water, filtered and the colouring matter precipitated with zinc chloride and brine. It forms a green iridescent mass which is at first sticky, but rapidly hardens. The substance dissolves in water with a magenta colour similar to that of toluylene red methochloride (p. 360). On analysis the zinc salt gave $N = 14·5$ per cent.; $C_{36}H_{42}N_8Cl_4Zn$ requires $N = 14·1$ per cent. The formula of the hydrochloride is therefore

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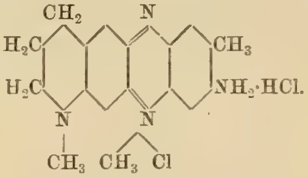
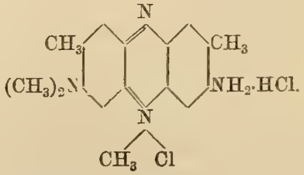
* 'Friedländer,' vol. 4, p. 376.



[89.] 2.*Dimethylamino*-7.*amino*-3.6.*dimethylphenazine methochloride.* — 16 parts of *p*-amino-dimethyl *o*-toluidine and 15 parts of *m*-dimethylamino *p*-toluidine were dissolved in 300 parts of water, 20 parts of conc. hydrochloric acid and 30 parts of 50 per cent. acetic acid, and cooled to 10° with stirring. Thirty parts of sodium dichromate in 400 parts of water were run in during 3 hours. A bluish-red coloration develops immediately and becomes more intense as the oxidising agent is added. The mixture was finally warmed on the water-bath for an hour, filtered and salted out with zinc chloride and sodium chloride. In this way the zinc chloride salt of the colouring matter is obtained. It was purified by solution in water, filtration and reprecipitation with salt solution. It could not be recrystallised from alcohol or dilute hydrochloric acid. The substance by analogy with the formation of tetramethyldiamino-phenazine (p. 361) has the following structure :—



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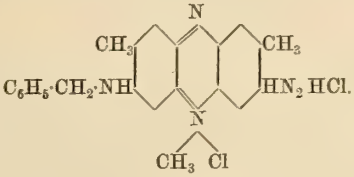
* 'Ber.,' vol. 16, p. 728 (1883).

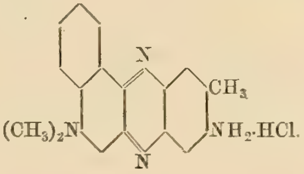
† Königs and Freer, 'Ber.,' vol. 18, p. 2388 (1885).

[90.] 2.*Benzylamino*-7.*amino*-3.6.*dimethylphenazine methochloride.* — The compound was prepared by heating together on the water-bath *p*-dimethylamino *o*-toluidine dissolved in alcohol with nitroso benzyl *o*-toluidine hydrochloride and hydrochloric acid as described in the preparation of the methylamino compound (p. 361). The colour developed passing from yellowish red to red, with a faintly blue tint. On concentration and cooling the colouring matter separated and was filtered and washed with ether. It has probably the following formula:—

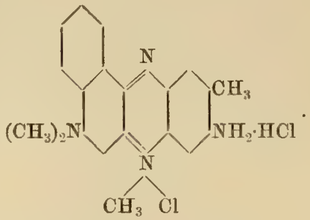


The zinc salt is obtained by precipitating the methochloride in solution with zinc chloride.

[91.] 7.*Amino*-6.*methyl*-2.*dimethylamino-napthophenazine hydrochloride.*— The substance was prepared as follows: 2·3 grms. of nitroso dimethyl *α*-naphthylamine hydrochloride were dissolved in 20 c.c. of hot glacial acetic acid, and to the solution was added a solution of 1·2 grms. of *m*-toluylene diamine in 20 c.c. of 50 per cent. acetic acid. 1 c.c. of conc. hydrochloric acid was then added and the mixture heated on the water-bath for 4 hours. A magenta colour developed rapidly and lost its bluish tint on continued heating, becoming gradually more crimson. It was finally boiled, diluted with 100 c.c. of water and the hydrochloride precipitated with brine. For purification it was redissolved in water and reprecipitated with brine. It has the following formula:—



[92.] 7.*Amino*-6.*methyl*-2.*dimethylaminenapthophenazine methochloride.* — The process was carried out as above, but instead of *m*-toluylene diamine the *p*-dimethylamino *o*-toluidine was used. 2·4 grms. of nitroso dimethyl *α*-naphthylamine hydrochloride were dissolved in 20 c.c. glacial acetic acid and added to 1·5 grms. of dimethylamino-toluidine in 20 c.c. 50 per cent. acetic acid, and 1 c.c. of conc. hydrochloric acid. The bright crimson colour develops rapidly, and after 3 hours' heating on the water-bath was separated and purified as in the previous case. The hydrochloride of the methochloride was even less soluble than the above hydrochloride, but is readily soluble in alcohol. The formula is



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2. Browning and Gilmour, 'Jour. of Path. and Bact.,' vol. 18, p. 144 (1913); see also Browning, Gilmour, and Gulbransen, 'Applied Bacteriology,' London, p. 65 (1918).
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The Action of "Peptone" on Blood and Immunity thereto.

By J. W. PICKERING, D.Sc., and J. A. HEWITT, Ph.D., B.Sc.

(Communicated by Prof. W. D. Halliburton, F.R.S. Received March 1, 1922.)

(From the Department of Physiology, University of London, King's College.)

The current view on the cause of the non-coagulability of the blood after the injection of "peptone" is that the injected material stimulates the liver (or the liver plus other organs) to form an excess of a substance named antithrombin. The liver is considered further to form antithrombin under normal conditions in amount sufficient to maintain the fluidity of the blood. The statement that "peptone" has little or no anticoagulant action on shed blood (except in high concentrations) is also generally accepted. Immunity to the anticoagulant action of "peptone" which follows its slow injection is commonly ascribed to hepatic activity.

The experiments recorded in this paper show that these views can no longer be held.

On the supposed Formation and Secretion of Antithrombin by the Liver.

Contjean (1), after ligature of the abdominal arterial trunks, and Gley and Pachon (2), after ligature of the lymphatics of the liver, found that injected "peptone" had no anticoagulant action. Starling (3) and Delezenne (4) could not confirm these observations, while Denny and Minot (5) have shown that repeated electrical stimulation of the cœliac plexus or its hepatic branches fails to increase the coagulability of the blood.

The first systematic attempts to prove that the liver secreted an antithrombic substance appear to have been made by Hédon and Delezenne (6), who, after establishing an Eck's fistula in a dog and, as far as possible, removing the liver, failed to reduce the coagulability of the blood by the intravascular injection of "peptone." These investigators concluded that something secreted by the liver was the cause of this result. In each of the two experiments described, some hours elapsed after the establishment of the fistula and before the liver was incompletely excised, and a further hour passed before the injection of "peptone." Delezenne (7) reported four similar experiments (of which only one is given in detail), and here also there is a considerable time-interval, in this case 5 hours, between the end of the operation and the injection of the "peptone." In the 26 minutes immediately following the injection of the "peptone" five samples of blood coagulated rapidly.

The common feature of these experiments is the great length of time during which the animals were under anæsthesia, leading to a decrease of oxygen (Buckmaster and Gardner, 8) and a corresponding increase of carbon dioxide in the blood.

Prior to the work of Hédon and Delezenne (*loc. cit.*), Lahousse (9) had shown that in peptone blood there is an extreme diminution of carbon dioxide, while Fano (10) and Wooldridge (11) had demonstrated that the passage of carbon dioxide through peptone plasma induces coagulation. Wright (12) has also shown that an increase of carbon dioxide in normal circulating blood augments its coagulability.

The following experiments* show that the retarded coagulability of peptone blood in animals intact, except for pithing, or in animals in which the liver is not acting, may be diminished or annulled by excess of carbon dioxide; the general result is that when the vitality of the animal is impaired this inevitably occurs with both moderate and larger doses of peptone.

In these and the subsequent experiments all animals were anæsthetised with A.C.E., pithed, and (except in Nos. 14–17) the anæsthetic was discontinued for at least a quarter of an hour before observations were made on the blood. All blood was withdrawn through evenly paraffined cannulæ. Artificial respiration (except in Nos. 14–17) was employed throughout. The animals were kept warm. The "commencement" of clotting was taken to be when the first change towards coagulation was observed; "completion" of clotting when the vessel containing the blood could be inverted without spilling. All glass vessels were cleaned with hot hydrochloric acid, caustic soda, alcohol and ether. Dust was as far as possible excluded. The percentages of "peptone" are necessarily approximate, since commercial specimens, as used by different investigators, vary enormously in composition. In the present work one preparation was employed throughout.

The results obtained in Experiments 1, 2 and 3 are expressed graphically. The points on the upper graph indicate "completion" of clot, those on the lower graph "commencement" of clot.

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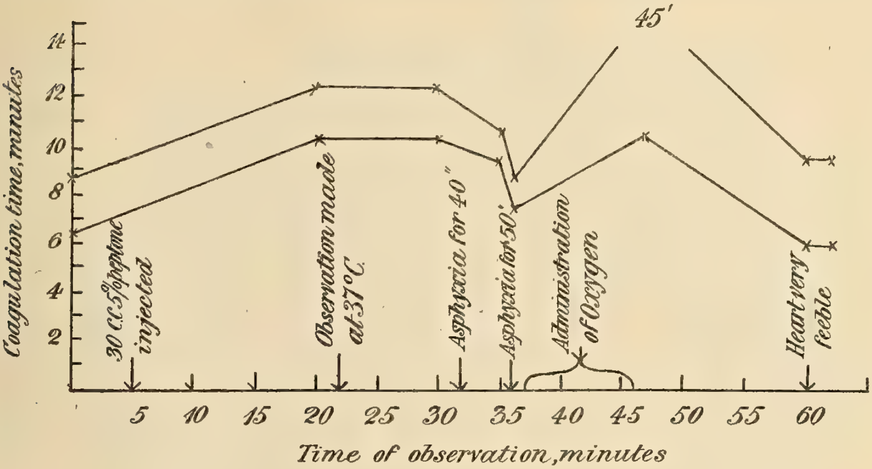
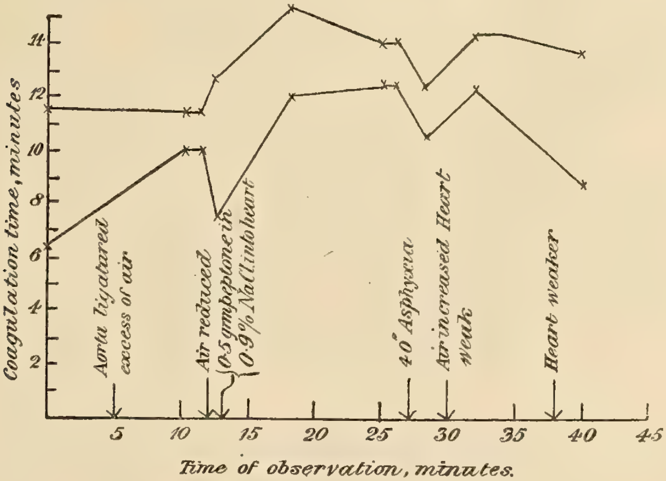
* Cats have been used in the experiments now recorded because their susceptibility to "peptone" lies midway between that of the dog, which is markedly susceptible, and the rabbit which is exceptionally resistant.

Experiment No. 1.—Pithed cat. Peptone injected into right jugular.



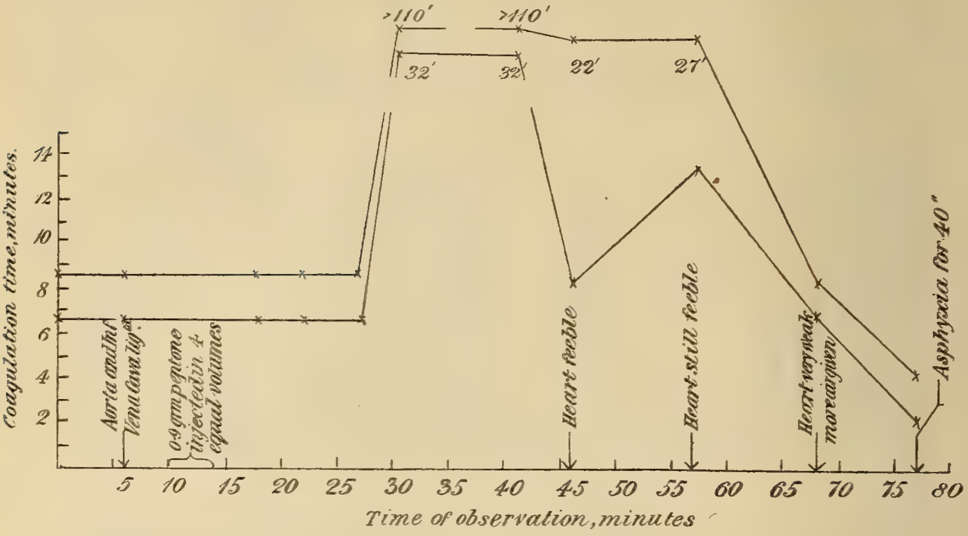
Note.—The clot at 47′ was very loose. From 60′ onwards the heart was weak.

Experiment No. 2.—Pithed cat. Aorta ligatured.



Note.—At 30′ and 41′, the blood was not completely clotted after 1 hour 50 minutes. *Post mortem* examination showed in this and in other experiments the ligatures intact.

Experiment No. 3.—Pithed cat. Aorta and inferior vena cava ligatured.



Experiment No. 4.—Cat, pithed. Artificial respiration. Aorta and inferior vena cava ligatured. 1·625 grm. of "peptone" was injected rapidly into the heart over a period of 4 minutes. Five minutes after the injection of the "peptone," coagulation time showed a retardation of 20 minutes in commencement of clotting, a complete gelatinous clot appearing 2 minutes 40 seconds later. This apparently liquefied, but further clots appeared in the liquid during the subsequent 2 hours, and the fluid was completely clotted on the following morning. While all other results have been repeated, this is the only occasion in these experiments where the phenomenon described in the dog by Doyon has been observed. Other samples of blood from this animal behaved similarly to those in Experiment No. 3, the increase in concentration of "peptone" used not causing any substantially greater inhibition than was evident in No. 3. The passage of carbon dioxide through the peptone blood from this animal gave typical clotting, as did dilution with distilled water.

The total time taken in No. 1 was 1 hour 10 minutes, in No. 2 1 hour 22 minutes, and, moreover, no anæsthetic was being respired. Hédon and Delezenne's experiments occupied "some hours," while in Delezenne's observation no less than 5 hours elapsed from the commencement of the operation till the observation of the coagulation time of the peptone blood. During these protracted periods a volatile anæsthetic was being administered and no attention was paid to the effect of a possible increase of the concentration of carbon dioxide in the circulation, under conditions liable to promote it. So far as the present writers are aware this criticism applies to all experiments in which "peptone" has been injected into animals with damaged or more or less excised livers.

Of the facts unexplained by the hypothesis that the secretion by the liver of antithrombin is the cause of the fluidity of normal circulating blood and of the retarded coagulability of peptone blood, it is noteworthy that the massive injection of thrombin does not produce thrombosis, Wooldridge (13). This was confirmed by Mellanby (14) who injected, without thrombosis, sufficient thrombin into a cat to coagulate 2 litres of blood. After the intravascular injection of thrombin, Davis (15) found the coagulation time of shed blood to be variable, sometimes slightly lengthened at other times shortened. Unfortunately Davis recorded as "coagulation times" only the time of completion of the clot. This may be a variable period when the times of commencement of clotting are approximately constant (Nos. 1–4). Moreover, Davis' variations in coagulation times are not greater than can be accounted for by alterations in the relative concentrations of carbon dioxide and oxygen in the blood shed. Apparently this and the corresponding hydrion concentration did not engage his attention. Davis explains the slightly decreased coagulability following the injection of thrombin by suggesting that thrombin produces an excessive secretion of antithrombin, while Howell (16) maintains that thrombin, or prothrombin is a hormone which stimulates the secretion of antithrombin. Davis frankly admits that if antithrombin exists in the circulation and is a genuine antibody, secreted as a protection against thrombin, then according to all analogies the presence of thrombin should lead to its production.

Nolf (17), however, denies that the intravascular injection of thrombin leads to the formation of antithrombin. Further doubt is cast on the accepted hypothesis by the recent work of Arthus (18), who observed that delay occurs in the coagulation of shed blood after the injection of the venom of *Crotalus adamanteus* into immunised, non-immunised, and even anaphylactic rabbits. This not only happened in whole animals, but in those in which the hepatic blood supply had been cut off.

Two views have been advanced in this connection, viz., that of Martin (19), and of Barratt (20), where the active fraction of snake venom is considered to be a true fibrin-ferment (thrombin), and that of Mellanby (21), that it is a kinase which, when rapidly injected, causes the rapid formation of thrombin. It is evident from the experiments of Arthus, that the "negative phase" of coagulation following either the rapid injection of minimal doses of venom, or the slow injection of larger doses, is not produced by the secretion by the liver of antithrombin, which combines with thrombin, either existing in, or produced by, venom. This conclusion falls into line with the recent observation of Pickering and Hewitt (22), that the slow addition, drop by drop, of tissue extract (nucleo-protein), prepared from kidney, to unsalted bird's blood *in vitro*, produces a " negative phase " in its coagulation, and is explicable by the suggestion that the " negative phase " of blood coagulation is a physical process involving an adsorptive union of the tissue extract, the substances forming the alkali reserve and fibrinogen, and is in its essence closely akin to the following *in vitro* reactions: the Dansyz reaction with the toxins and antitoxins of diphtheria and ricin (Dansyz, 23), the variations in the toxicity incidental to the fractional neutralisation of arsenious acid by ferric hydroxide (Hewlett, 24), the " negative phase," or temporary inhibition of the precipitation of gelatin by the slow addition of either alcohol or ammonium sulphate, and to the similar phenomena following the addition of electrolytes to certain inorganic sols (Spring, 25, Höber and Gordon, 26, Paine, 27, Galecki, 28, Burton, 29).

Arthus concluded that in poisoning by proteotoxins, the liver is either not the organ which produces anti-thrombin, or is not the only organ. Doyon (30) immediately perceived the significance of Arthus' work, and, adopting his methods, injected " peptone " into dogs in which the hepatic circulation had been ligatured. Again, no attention was paid to the probable increase of carbon dioxide in the blood during the experiments in which anæsthetics were employed. Doyon states that, in the cases he observed, the speed of clotting of shed blood was normal, but the clots were soft and completely dissolved on standing, so that the blood again became fluid. This result Doyon ascribed to the action of anti-thrombin formed in parts of the body other than the liver, and reverted to Contjean's views (*loc. cit.*), which have also been supported by Popielski (31).

It is, it is thought, clear that Doyon's clots are not true fibrin clots, as such do not re-dissolve *in vitro*. In a recent paper (Pickering and Hewitt (*loc. cit.*)) evidence has been brought forward that the first stage of coagulation of mammalian and frog's blood, surrounded by oil, is the formation of a reversible gel, which is soluble in excess of tap water. In the case of frog's blood this stage may, when undisturbed, persist for several hours, but if thromboplastic material is added to it a typical clot is formed in a few minutes. It therefore appears possible that " peptone," as administered by Doyon with the liver out of circulation, retarded the coagulation of the blood so that it remained in the first stage of the process. Only in one of the series of experiments now recorded, in which " peptone " was injected into both intact cats and into cats deprived of hepatic circulation, has any evidence been noted of the temporary formation of a gel which dissolves on standing (No. 4). It has consequently been impossible to investigate any relationship to hydrion concentration of this transient but significant phenomenon.

The earlier observations on the effect of the injection of "peptone" into animals with either impaired or partly excised livers, have formed the basis for a vast superstructure of hypotheses. They remain crucial only so long as the anticoagulant action of "peptone" has not been observed in animals when the liver is not acting.

Employing a somewhat simpler operative technique to deprive the liver of circulating blood with a view of avoiding the prolonged interval before the injection of "peptone" (with the consequent accumulation of carbon dioxide), the results given in experiments, Nos. 2, 3 and 4 above, demonstrate decisively that *retardation of coagulation of blood consequent to the rapid intravascular injection of moderate amounts of "peptone" can be produced under conditions which preclude the participation of the liver.*

Stress is laid on the importance of working with pithed animals, and in allowing at least 15 minutes to elapse after pithing and the discontinuance of the anæsthetic before the injection of "peptone," so that any action of the anæsthetic on the coagulability of the blood may be eliminated. It is also advisable to regulate the air supply to the animals so as to maintain, as long as possible, normal oxygenation.

On the action of "peptone" on blood in vitro.

The well known observation of Schmidt-Mulheim (32) that very large quantities of "peptone" are necessary to retard the coagulation of blood *in vitro* is commonly regarded as showing that "peptone" has no specific action on shed blood. Among the earlier observers, Afanassiew (33), however, stated that blood shed direct from the artery of a dog, without exposure to the air, into 1·225 — 1·5 per cent. "peptone," and kept at 40°, remained fluid. Unfortunately the details of the experiment are meagre. Pollitzer (34) stated that 2 per cent. of heteroalbumose inhibited blood clotting *in vitro*, while Halliburton (35) recorded that the same substance also delayed the coagulation of salted plasma. Working with lymph from the thoracic duct which clotted, at room temperature, in from 10 to 15 minutes, Shore (36) found that when relatively large quantities of "peptone" were added *in vitro* the clotting was as rapid as in pure shed lymph, but if smaller amounts of "peptone" were used retardation occurred. In one experiment in which the amount of "peptone" was 0·0377 per cent., the lymph remained fluid for 24 hours.

Camus and Gley (37) reinvestigated the effect of adding "peptone" to shed blood, and stated that from eleven to fifteen times more "peptone" is required to produce retardation of clotting *in vitro* as compared with the amount necessary to give this result *in vivo.* Thus the original views of the non-specificity of the action of "peptone" on shed blood gained general acceptance.

The present authors find that when blood is shed into "peptone" without precautions to preserve it from changes towards coagulation, as in the earlier work, then anticoagulant action is absent, except when the "peptone" is present in relatively large amount. The following experiments show that different results are obtained when blood is shed through an evenly paraffined cannula from an animal respiring air.

No. of experiment	5.		6.		7.		8.		9.		10.	
Percentage of "peptone" in blood	p.c. 0·3		p.c. 0·325		p.c. 0·6		p.c. 1·0		p.c. 1·0		p.c. 3·5	
	′	″	′	″	′	″	′	″	′	″	′	″
Coagulation times of control blood	7	45	5	55	7	45	7	45	5	55	7	25
	11	45	8	10	11	45	11	45	8	10	8	45
Coagulation times of "peptone" blood	13	20	45	20	16	30	14	45	21	4	?	
	16	40	48	50	20	10	23	40	23	40	?	
Coagulation times of "water" blood	8	10	6	5	8	10	8	10	8	10	6	5
	10	15	7	55	10	15	10	15	10	15	7	55

Notes.

1. "Water" blood is normal blood to which a volume of water or in No. 6 of 0·9 per cent. NaCl equal to that of the peptone has been added.
2. The upper of the two coagulation times is that of "commencement," the lower that of "completion" of clotting.
3. The blood was arterial and was withdrawn into glass vessels through paraffined cannulæ. The animals were pithed and were breathing air only.
4. The "peptone" in Experiment No. 6 was dissolved in 0·9 per cent. sodium chloride; in all others in this series in distilled water.
5. Room temperature was 13–14° C.
6. The "peptone" blood in No. 10 remained fluid during the next day.

Experiment No. 5 shows that blood withdrawn from the carotid of a cat through a carefully paraffined cannula into a clean glass vessel, and there mixed immediately with "peptone" dissolved in distilled water, exhibited a distinct retardation of clotting. This delay amounted to 5 minutes 25 seconds in the time of commencement, and 4 minutes 55 seconds in the time of completion of clotting; the amount of "peptone" in the blood was only 0·3 per cent. As is seen from experiments 7, 8, 9, and 10 with greater concentrations of "peptone," the inhibition of clotting increased. When solution is effected in 0·9 per cent. sodium chloride as in Experiment No. 6, the inhibition of coagulation is still more marked. Thus with a concentration of 0·325 per cent. of "peptone" in the blood a delay of 39 minutes 25 seconds in the commencement of clotting was observed, while the delay in completion was 40 minutes 40 seconds. The addition of corresponding amounts of 0·9 per cent. sodium chloride to shed blood did not produce any appreciable variation in the coagulation time. A comparison of Experiments Nos. 8 and 9 with No. 6 shows that the retarding effect of a concentration of 1 per cent. of "peptone" dissolved in distilled water was actually less than that of 0·325 per cent. dissolved in normal saline. This result falls into line with the observation of Howell (38) that while dilution of peptone plasma with water induces coagulation, dilution with 0·9 per cent. sodium chloride has no such effect.

In the next three observations, endeavours were made, with success, to prolong the anticoagulant action of small amounts of "peptone" *in vitro* by reducing the disturbance to the colloidal complexes of the plasma incidental to the shedding of blood.

	Control blood on glass.	Control blood on paraffin.	0·3 p.c. peptone blood on paraffin.	0·3 p.c. peptone blood on paraffin.	0·6 p.c. peptone blood on paraffin.
	′ ″	′ ″	′ ″	′ ″	′ ″
Time of adding "peptone"...	0 0	0 0	0 0	0 0	0 0
Time of "commencement" of clotting	8 55	24 20	32 40	34 45	45 20
Time of "completion" of clotting	12 15	27 40	107 30	136 0	135 0
No. of experiment	—	—	11	12	13

Notes.

In Nos. 12 and 13, after 171 minutes there was still some unclotted blood.

Many experiments of this nature have been carried out in this connection. Only one is given.

Experiments.

In these experiments, blood from the carotid through a paraffined cannula was received into paraffined vessels, as free from dust as possible. With 0·3 per cent. of "peptone" (dissolved in 0·9 per cent. sodium chloride) in the blood, again mixed *in vitro*, the delays in the commencement of clotting exceeded 23 minutes, as compared with the coagulation time on glass, a result which was not more marked than was obtained with 0·325 per cent. of "peptone" dissolved in 0·9 per cent. sodium chloride, mixed in an uncoated glass vessel. The distinctive difference between the results on glass and paraffined vessels is that, in the former, coagulation, once it has commenced, proceeds rapidly, and is completed in a few minutes, while in the latter it is a relatively slow process, occupying from 1 to 2 hours. In some cases, indeed, the first formed small clots are exhibiting syneresis (contraction), while other clots are still forming. Thus, in the less disturbed conditions of the paraffined vessels is the continuous inhibitory action of the "peptone" made evident.

A comparison of the coagulation times of pure blood on paraffined surfaces with those of blood mixed with "peptone" on similar surfaces shows less relative retardation in the time of commencement of clotting than is evident when the coagulation of pure and peptone blood in uncoated vessels is compared. The relative retardation in the time of completion of the clots is much more marked when blood mixed with "peptone" is in paraffined vessels than when it is contained in glass vessels not so treated.

In this connection, the partial failure of one experiment is noteworthy. The blood contained 0·8 per cent. of "peptone." It did not commence to clot until 31 minutes after the mixing of the blood and "peptone," but coagulation was complete 2 minutes later, a firm clot being formed which could be inverted without spilling.

This is a marked contrast to Experiments 11, 12 and 13, and cannot be ascribed to the higher concentration of "peptone" used, as typical slowing of clotting has been obtained after the appearance of the first clot in a number of cases where this and higher concentrations of "peptone" have been employed. In the specific case referred to, examination of the vessel containing the peptone blood revealed a small defect in the paraffin lining, with adherent fibrils to the exposed glass. Clotting had started from this point, and had spread through the liquid.

Attention is drawn to Experiment 13, where the "peptone" was 0·6 per cent. of the mixture; here clotting did not commence till 45 minutes 20 seconds, and was completed only as a semi-gel 2¼ hours after mixing. A small amount of the fluid was withdrawn by a pipette before coagulation was complete, and carbon dioxide passed through it. Clotting was observed after 10 minutes. This result was also obtained by dilution with distilled water. Other observations gave similar results. During the progress of the delayed clotting, portions of what remained fluid were, from time to time, withdrawn, and mixed with one-fourth of their volume of saturated ammonium sulphate. A precipitate of what was probably fibrinogen was always obtained (McLean, 39), till, at the end of the coagulation process, when the clots appeared to be complete, tests of the residual fluid yielded no precipitate. The mixture of "peptone" and blood *in vitro* thus behaved precisely as the blood peptonised *in vivo*.

Schmidt-Mulheim (*loc. cit.*) gives the dose of "peptone" necessary to produce incoagulability of circulating blood of dogs as from 0·3 to 0·6 grm. per kilogramme. Pollitzer (*loc. cit.*) found cats to be more resistant. Taking an animal weighing 3 kgrm., it may be assumed to contain 200 c.c. of blood. If 0·3 grm. per kilogramme of "peptone" is injected, the percentage in the blood is 0·45, or 0·9 if the larger quantity is used.

If, from a pithed cat which is breathing only air for at least 15 minutes, arterial blood is obtained through a paraffined cannula with only smooth surfaces exposed to the blood, then less than Schmidt-Mulheim's minimal dose for the dog will produce, in the more resistant cat, an unequivocal anticoagulant action *in vitro*, even in uncoated glass vessels. When the concentration of "peptone" is increased, but is still 33 per cent. less than Schmidt-Mulheim's larger amount, the results *in vitro* are parallel to those following the injection of "peptone" into the circulation of the living animal.

These data warrant the following conclusions:—

1. "Peptone," even in small quantities, acts directly on the constituents of the blood.

2. The apparent difference found by other observers between the action of "peptone" *in vivo* and its action *in vitro* is due to the changes towards clotting which take place in blood shed without special precautions to preserve its surface conditions.

3. It is superfluous to assume that the anticoagulant action of "peptone" *in vivo* is due to the secretion of antithrombin either by the liver or by the endothelial cells of the vascular system.

On the Supposed rôle of Leucocytes in the Anticoagulant Action of "Peptone."

Delezenne (40) considered that the first action of "peptone" on blood is leucolysis yielding two substances: *leuconuclein* which hastens coagulation and *leucohistone* which retards it. These both circulate through the liver where the former is retained. The latter remains circulating. Several workers including Halliburton and Brodie (41), Falloise (42) and Dastre and Studel (43) however deny that "peptone" on injection causes leucolysis. Even if this process does occur there appear to be no grounds other than pure speculation for the supposed liberation of these substances into the blood stream.

Nolf (44) maintained that under normal conditions an *unknown* substance is produced by the leucocytes which acts upon the endothelium of the liver and stimulates it to secrete antithrombin. The injection of "peptone" enhances this process and the excess of antithrombin accounts for the retarded coagulability of peptone blood.

Having demonstrated that the liver is not necessary for the production of the anticoagulant action of "peptone," it becomes of interest to enquire whether the leucocytes are concerned in this phenomenon. To this end Experiments 14–20 were devised.

Cats were bled through paraffined cannulæ into paraffined centrifuge tubes in ice and without delay these were centrifuged at 4° C., after which the tubes were again surrounded by ice till required. By this method a clear fluid mammalian plasma was obtained unaltered by foreign substances and which had made but little, if any, progress towards coagulation. At the temperature mentioned leucocytes do not exhibit amœboid movement and it may be assumed that all secretion by them is inhibited. To this plasma "peptone" was added in concentrations varying from 0·3–1·2 per cent. of the total volume of "peptone" and plasma and the mixture was observed at room temperature in paraffined vessels. In all cases except one, the typical anticoagulant action of "peptone" was evident. A study of the protocols shows that the results are essentially the same as when "peptone" in similar concentration is either added to shed blood at laboratory temperatures or is intravascularly injected into whole animals or into animals where the liver is out of circulation. Evidence is thus forthcoming that *leucocytes play no part in the inhibition of coagulation which follows the rapid admixture of blood and "peptone."*

	Control plasma on paraffin.		Control plasma on glass.		0·3 p.c. peptone plasma on paraffin.		0·6 p.c. peptone plasma on paraffin.		0·9 p.c. peptone plasma on paraffin.		0·9 p.c. peptone plasma on paraffin.	
	'	"	'	"	'	"	'	"	'	"	'	"
Time of adding "peptone" ...	0	0	0	0	0	0	0	0	0	0	0	0
Time of "commencement" of clotting	8	0	5	0	7	40	12	5	19	30	16	0
Time of "completion" of clotting	10	10	8	20	9	10	54	50	No end point		27	0
No. of experiment	—		—		14		15		16		17	

Notes.

Plasma obtained from animal respiring A.C.E. and air.

1. In No. 16 clotting was not typical; small clots were formed.
2. In No. 17 the gelatinous clot appeared to liquefy after 29 minutes; 10 minutes later all was liquid except one small clot. Syneresis is not excluded.
3. The residual fluids from 16 and 17 gave, next morning, no precipitate when fifth saturated with ammonium sulphate.
4. No. 14 is the only anomaly in a large number of observations.

A comparison of Experiments 14–17 with Nos. 18–20 illustrates the difference between the action of "peptone" on plasma from an animal under complete anæsthesia (A. C. E.) and the action of similar quantities of "peptone" on blood from a pithed animal 15 minutes after the discontinuance of the anæsthetic.

There is consistently less "peptone" inhibition of clotting when the animal is under anæsthesia at the time the blood is obtained. Early in these experiments it was evident that the shed blood of anæsthetised animals was

	Control plasma on paraffin.		0·6 p.c. peptone plasma on paraffin.		0·9 p.c. peptone plasma on paraffin.		1·2 p.c. peptone plasma on paraffin.	
	′	″	′	″	′	″	′	″
Time of adding peptone	0	0	0	0	0	0	0	0
Time of "commencement" of clotting	15	0	34	45	50	20	24	30
Time of "completion" of clotting	23	50	41	45	51	20	30	0
No. of experiment	—		18		19		20	

Notes.

1. Repetition of Nos. 18 and 19 gave the same results.
2. In No. 20, after 20 minutes the gel appeared to liquefy, due probably to syneresis.
3. Plasma was obtained from animal respiring air.

more prone to coagulate rapidly than that of pithed animals 15 minutes after respiring only air. For this reason, except for the experiments cited above, all observations not on pithed animals were discarded.

In both the last two series of experiments typical syneresis was exhibited in the clots formed. The view that blood platelets are necessary for the production of syneresis (Bordet and Delange, 45) may be mentioned.

The Influence of Speed of Injection on the Action and Immunity to the Action of Peptone on Blood.

Fano (46) found that, if "peptone" is injected slowly into the circulation, it has not any anticoagulant action on the blood, and later Nolf (*loc. cit.*) showed the concentration of "peptone" as well as the speed of its injection to be the determining factors either in the production of anticoagulant action or complete or partial immunity thereto. Nolf explained the immunity following minimal doses injected rapidly and larger doses injected slowly by suggesting that leucocytes are not acted upon by "peptone" if delivered sufficiently slowly, and that they become accustomed to increasing doses. The leucocytes, he believes, under these conditions fail to produce the *unknown* substance which stimulates the liver to secrete either antithrombin or fibrinolysin. The experiments described in the preceding section demonstrate that leucocytes are not essential for the production of the anticoagulant action of "peptone" *in vitro*, and there appears to be no valid reason for assuming their presence to be necessary for the production of the same effect *in vivo*, when the concentrations are strictly comparable.

In sharp distinction to the generally accepted view is that of Mellanby (47), who maintained that fluidity of peptone blood is due to the secretion of an excess of alkali by the liver, under the influence of a stimulus of injected "peptone," and the "peptone" immunity is caused by the temporary disappearance from the liver of the excess of alkali secreted under the toxic stimulus.

The next experiment, No. 21, shows that if relatively large amounts of "peptone" are injected slowly, in minimal doses, over a long period of time, into a cat in which the circulation through the liver has been prevented, then, as in the intact animal, no anticoagulant action is to be obtained.

Experiment No. 21.—Cat, 3¼ kilos. Pithed, artificial respiration. Aorta ligatured beyond the coronary arteries, inferior vena cava above the diaphragm.

Over period of 1 hour 10 minutes 1·8 grm. of "peptone" in 0·9 per cent. NaCl was injected in nine equal amounts, each injection being 2·5 c.c. in volume and being made at approximately equal intervals of time.

The coagulation times of the blood was taken at frequent intervals during the injection of the "peptone" and at no time showed any delay.

Control Blood on Glass.—Commencement of clotting, 9 minutes; completion of clotting, 12 minutes 10 seconds.

The total amount administered was 1·8 grm., and as only about one-fourth of the animal's blood was circulating, the concentration of "peptone" in the blood at the end of the experiment may be estimated at 3·6 per cent., an amount much in excess of that required to produce anticoagulant action both in whole cats and in cats in which the liver is not acting, provided that such an amount is injected slowly. Moreover, reference to Experiment No. 10 shows that a concentration of 3·5 per cent. of peptone in the blood *in vitro* produced incoagulability over a period of 24 hours. Assuming in the *in vivo* experiment mentioned above that the quantity of blood dealt with was 50 c.c., a concentration of "peptone" exceeding that required to produce prolonged incoagulability *in vitro* was injected without anticoagulant effect.

Some other explanation must therefore be sought for this immunity to the slow injection of "peptone" than is supplied by the divergent views of Nolf and of Mellanby. These hypotheses have one point in common, they assign the cause of immunity to hepatic secretion, and it is precisely this point of both theories which is shown to be untenable by the experiments given above. Whether the alkali reserve or the hydrion concentration of the plasma is concerned in these occurrences must be left for future determination. There are, however, some indications which point in this direction. Fano (*loc. cit.*) showed that peptone blood could be clotted either by the passage through it of carbon dioxide or by neutralisation by acid. Earlier in the present paper the influence of the concentration of carbon dioxide in circulating blood on the action of "peptone" has been emphasised. It is noteworthy that Mills (48) has recorded that a diminution of the alkali reserve occurs *pari passu* with the coagulant action arising from the intravenous injection of lung extract. It is well known that the advent of asphyxia will determine the appearance of thrombosis after the injection of such amounts of kidney nucleoprotein as, in the presence of normal amounts of carbon dioxide, tend to produce incoagulability or immunity. In like manner Halliburton and Pickering (49) showed that an increase in the concentration of the carbon dioxide of the blood will annul the "negative phase" resulting from the injection of minimal doses of these synthesised substances which have been shown to be intravascular coagulants (Pickering, 50).

Still more significant is the correlation of all these "negative phases" and temporary immunities to the factors of the speed of action and concentration of the toxic substances. In this respect they are similar to the "negative phases" which occur *in vitro* alluded to earlier in this paper.

There is a physical or physico-chemical interpretation indicated.

An application of the theorem of Le Chatelier (51) may provide a more satisfactory explanation than is afforded by current hypotheses.

On Experiments with perfused Livers.

It is commonly held that perfusion of the liver affords evidence of the secretion of antithrombin by that organ. Delezenne (52) and later Nolf (53), who had doubtful results, found that perfusion of the liver with a mixture of defibrinated blood and "peptone" gave an incoagulable liquid which, when added to shed blood, retarded its coagulation. Apparently these workers were unaware of earlier observations of Pavloff (54), who had found that the circulation of a mixture of defibrinated and normal blood through a heart-lung preparation yielded a fluid which remained uncoagulated for several days. Work is now in progress which indicates that certain products of autolysis may act as anticoagulants, and in view of Pavloff's experiments it is superfluous to assume that Delezenne's results were due to hepatic activity. Doyon (55) connected the carotid of one dog with the portal vein of another, and examined the coagulation times of blood from the inferior vena cava. With young fasting dogs the first perfusates coagulated normally, but later samples showed delay in coagulation. With adult dogs, even after fasting, no retardation of clotting was observed. It is well known that the blood of embryos is resistant to clotting and these isolated experiments only afford evidence of a similar phenomenon in the blood of young fasting dogs.

Summary and Conclusion.

1. The retardation of the coagulation of the blood resulting from the rapid intravascular injection of "peptone" into either whole cats or into cats with the liver out of circulation is reduced or annulled by increase of carbon dioxide in the blood.

2. The anticoagulant action of "peptone," if annulled by an increase of carbon dioxide, can be restored by administration of oxygen or of excess of air.

3. In pithed animals respiring air, the retardation of coagulation of shed blood following the rapid injection of moderate amounts of "peptone," can be produced when the liver is out of the circulation.

4. Previous failures to obtain this result are due to attention not having been paid to the increase of carbon dioxide in the blood of animals with impaired vitality following operation and prolonged narcosis.

5. With suitable precautions to preserve the surface conditions of the blood, typical "peptone" delays of coagulation can be obtained *in vitro* with quantities of "peptone" no greater than are required to produce a similar effect in the living animal.

6. If special precautions are not taken to preserve the surface conditions of shed blood, admixture *in vitro* with moderate amounts of "peptone" causes no appreciable anticoagulant effect.

7. Leucocytes play no part in the anticoagulant action of "peptone" on blood.

8. It is superfluous to assume that the anticoagulant action of "peptone" on blood is due to hepatic secretion of an excess of alkali under the toxic stimulus.

9. It is unnecessary to assume that the anticoagulant action of "peptone" is due to the secretion of antithrombin, either by the liver or by other cells of the body.

10. Typical immunity to the action of "peptone" on blood can be obtained by injecting maximal amounts, in repeated small doses, into animals with the liver out of circulation.

11. The current hypotheses on "peptone" immunity are shortly discussed, and reasons given for not accepting them. Immunity to "peptone" appears to be a physical process akin to adsorption.

12. Experiments on the perfusion of the liver do not show that antithrombin is normally secreted by that organ.

13. If the foregoing conclusions are correct, it follows that in the interpretation of the coagulation of the blood it is unnecessary to assume the existence of *antithrombin*, *pro-antithrombin*, and *anti-prothrombin*.

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14. If antithrombin is not a normal secretion of the liver, some explanation of blood coagulation, other than the current "thrombin theories," is necessary.

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Active Hyperæmia.

By D. T. Harris (Beit Memorial Research Fellow).

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(From the Institute of Physiology, University College, London.)

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1. Introduction.

So conflicting are the statements made by physiologists and pathologists alike, relating to the mechanism by which "an increased afflux of blood to a part" is brought about, that it was felt desirable to investigate this important phenomenon. Let us first consider the present state of knowledge regarding the possible factors involved.

(i) *Vaso-constrictor Nerves* (neuro-paralytic hyperæmia).—The existence of vaso-constrictor nerves was first demonstrated in the cervical sympathetic by Claude Bernard (1852), and independently by Brown-Séquard. The interruption of the normal stream of impulses from the vaso-motor centres along these nerves gives rise to a "neuro-paralytic hyperæmia," such as is met with surgically in wounds of the neck, or in lesions of the spinal cord. During the normal functional activity of a particular organ, reflex excitation of the vaso-constrictor nerves of the resting organs liberates a maximum blood supply for the working organ; this is a regulating mechanism of the central nervous system which ensures a more efficient functional hyperæmia of the working organ (Lovén, 1866; Bayliss, 1920).

(ii) *Vaso-dilator Nerves* (neuro-tonic hyperæmia).—The first evidence for the existence of vaso-dilator nerves was supplied by Claude Bernard (1858), who found that stimulation of the chorda tympani caused not only secretion by the submaxillary gland, but also dilatation of its blood vessels. Vulpian showed the reddening of the tongue upon stimulation of the lingual nerve, and proved that the fibres concerned came from the chorda tympani. Goltz had noticed that whereas stimulation of the sciatic nerve, when freshly cut, caused pallor of the skin of the toes and a lowering of temperature as measured by a thermometer placed between the toes, the same stimulation of the nerve two days after section caused reddening and a rise of temperature. He concluded that vaso-dilator, as well as vaso-constrictor nerves, existed in the sciatic, and that they resisted degeneration for a longer time than the vaso-constrictors. Bernstein showed that the vaso-dilator fibres in the sciatic could be excited independently by means of slow rhythmic shocks, especially in a leg which had been previously cooled.

The remarkable nature of the vaso-dilator supply to the limbs, skin of the trunk, and probably of the ears and face, and to the intestine, was not explained until 1901, when Bayliss investigated vaso-dilation in these regions produced by stimulation of the peripheral cut ends of the corresponding dorsal roots. The work of Head and Campbell (1900), on herpes zoster, shows this disease to be due to irritative lesions of the dorsal root ganglia, which give rise to abnormal impulses in an efferent (anti-dromic) direction along the sensory fibres to the skin; whether stimulation of vaso-dilator nerves can produce blisters has not yet been proved. Ninian Bruce (1910) showed that the "neurotonic hyperæmia" (Adami), produced in the conjunctiva by an irritant like oil of mustard, could be prevented by first paralysing the sensory nerves with cocaine, or by section and degeneration; this was explained as an "axon reflex," since the hyperæmia depends on the integrity of the sensory fibre.

(iii) *Vaso-dilator Substances* (a local hyperæmia).—It is generally held that the hyperæmia of inflammation arises from the action of physical and chemical agents directly upon the blood-vessel wall. The great similarity between a wheal produced by the lash of a whip, for example, and the effects of a local introduction of histamine (β-iminazolyl-ethylamine) into a lightly scarified area of skin lends support to this view, since the depressant action of histamine locally upon the capillary wall is definitely known (Dale and Richards).

What are the possible chemical vaso-dilator substances which may exert a "depressor" effect on the plain muscle or endothelium of the blood vessels of an organ so as to ensure a greater blood supply during activity? Two possibilities are presented:—

α. *Acid Metabolites*: The more or less completely oxidised products, *e.g.*, CO_2 and lactic acid, derived from the energy-producing food-stuffs.

β. *Basic* split products resulting from the wear and tear of the cell protoplasm.

(iv) *Functional hyperæmia.*—With this brief survey of the factors which produce vaso-dilation, we may embark upon the controversial question of the means whereby the functional hyperæmia of an active organ is brought about. Leaving out of the discussion the alterations in general blood pressure (*vide* (i)), the issue lies between neuro-muscular vaso-dilation and the automatic vaso-dilation produced by metabolites. On the one hand, among our best authorities we have some who regard functional vaso-dilation as a simple local effect of the metabolites resulting from activity, just as the heat evolved results from the chemical changes of activity, and there is, according to them, as little need to presume the existence of special vaso-dilator nerves as there is to suppose the presence of caloric nerves.

Barcroft (1914, p. 145) found that during stimulation of the sympathetic fibres in the submaxillary gland (cat) by means of adrenalin, the vaso-constriction ordinarily produced by adrenalin on blood vessels is more than counterbalanced by the action of the vaso-dilator substances produced when the gland secretes.

On the other hand it is well known that stimulation of the chorda tympani in an atropised submaxillary gland causes a vaso-dilation in the total absence of secretion; Barcroft explains this by advancing the possibility of lower grades of cellular activity, without external secretion occurring in such cases, and he actually found that during stimulation there was an increased oxygen intake in the atropised gland. An examination of his figures (p. 147) shows, for example, equal metabolic rates for a 4- and 2-fold blood flow:—

Per cent. increase in oxygen	55	55
Per cent. increase in blood flow	333	102

Here there must be, as Bayliss (1920) points out, a factor in addition to metabolites called into play; the hyperæmia in the last experiment can hardly be caused by metabolites arising from activity of such "subliminal degree"; it would appear that the neuro-muscular mechanism is also excited to activity. Anrep (1916) has shown that when secretin used to stimulate the pancreas is free from the depressor substance, there is little or no sign of vaso-dilation in the gland associated with secretin; this is also seen in some of the experiments of Barcroft and Starling (1904) on the gas exchanges in the pancreas. Thus, during the normal rate of activity in glandular organs, the metabolites are quickly removed and never reach a high concentration. This is not the case with skeletal muscle where a prolonged contraction compresses the blood vessels and banks up the metabolites. Asher (1910), by means of minute doses of sodium fluoride, was able to paralyse the secretory activity of glands without eliminating the vaso-dilator response, from which he concluded that vaso-dilation could occur independently of metabolites.

The present investigation is an attempt to clear up these fundamental problems:—

a. To obtain evidence, if possible, for the undoubted existence of vaso-dilator nerves.

b. To determine the relative part played by metabolites and vaso-dilator nerves in functional hyperæmia.

c. If vaso-dilator nerves actually exist and are not an essential to functional hyperæmia, what, then, is their function?

2. METHODS.

i. Measurement of vaso-dilation: *a. direct* blood flow;
b. indirect plethysmograph.
ii. Estimation of metabolites: *a. alkali reserve;*
b. lactic acid.

The preparation: The tongue, as pointed out by Anrep, possesses unique advantages for the examination of these problems: the individuality of function of its several nerves, which run separate courses, constituting an easy and very certain means of determining the cause of hyperæmia. The functional activity is almost purely muscular, since the total mass of lingual glands is of comparatively insignificant amount. Dogs were found to be the most convenient animals for these experiments which involved a good deal of operative procedure, the accessibility of the structures in these animals reducing injury to the tissues to a minimum. A preliminary dose of morphia was given and complete anæsthesia maintained by means of chloroform and ether; in the few cases in which curare was injected decerebration preceded. The lingual and hypoglossal nerves were exposed on both sides, and the blood pressure was recorded in the femoral artery.

Venous outflow: A cannula was placed in the anterior transverse vein connecting the two lingual veins, with ligatures and artery forceps so placed as to allow of the collection of samples of blood from the lingual vein while permitting the normal flow in the interval. No anti-coagulant was used, but all tubes and cannulæ were paraffined. The rate of flow was noted by a drop recorder.

For the estimation of lactic acid, 20 c.c. of blood was collected directly into 0·5 per cent. KH_2PO_4; for the determination of the P_H and alkali reserve, the blood was collected under paraffin, oxalate being used as an anti-coagulant. By attaching the hyoid bone to an isometric muscle-lever, the tongue muscles could be made to work against a resistance and develop a measurable tension (*e.g.*, fig. 5).

Plethysmograph of the tongue: The changes in volume of a curarised tongue were originally studied by Anrep (senior) and Cybulski (1884), and later with the same methods by Piotrowski (1893). For the purposes of the present investigation curarisation could not be adopted for maintaining the tongue, or rather part of it, placidly in the plethysmograph. In order to permit the normal production of metabolites, a technique had to be devised which enabled one to take reliable records of an active muscular organ. Many failures were encountered before the following arrangement was arrived at of accommodating the whole tongue in the plethysmograph and preventing its moving in and out during contraction.

The genio-hyoid muscles were detached from the symphysis of the jaw and the mucous membrane of the floor of the mouth was cut through in close contact with the alveolar margin as far back as the anterior pillars of the fauces. The tongue was now drawn out ventralwards, all bleeding arrested by ligature and cautery, and the cut edges of the mucous membrane sewn together round the genio-hyoid muscles, carefully avoiding pressure on the lingual vessels and nerves; the whole tongue could thus be placed in the plethysmograph. To prevent traction on the root of the tongue from without, a tracheal canula was inserted, the trachea being completely cut across; all muscles coming up to the hyoid were cut between ligatures, and all branches of XII, other than those to the tongue, were severed; finally, to completely immobilise the larynx and hyoid, a steel rod placed through the mouth into the larynx, and emerging from the upper end of the transected trachea, was firmly clamped to the same stand as the plethysmograph.

A plethysmograph of circular cross-section was selected which just fitted the tongue; it was fitted with a cuff of membrane rendered air-tight, yet soft, by means of vaseline. When adjusted it was covered with cotton-wool and connected to a piston recorder: time was allowed for temperature adjustment.

3. The Vaso-constrictor Nerves.

The vaso-constrictor fibres are supposed to be derived from the cervical sympathetic and run in the hypoglossal nerves: the sympathetic fibres for the *muscles* of the tongue seem to run, not in the hypoglossal, but in the chorda tympani (Boeke, 1921).

The right vago-sympathetic trunk was ligatured in the neck, cut below the ligature, and the vagal portion was transected at its emergence from the skull. Faradisation of the peripheral end of the R. sympathetic affected the venous outflow in the manner shown in fig. 1.

During stimulation the drops are seen to become less frequent and on removal of the stimulus the still further decrease indicates that either the vessels immediately relax and accommodate the first outflow of blood, or there is a marked "after effect" following the stimulus.

Faradisation of the hypoglossal nerve in the curarised animal verifies the fact that the vaso-constrictors run in this nerve (fig. 2), as was first clearly shown by Anrep (senior).

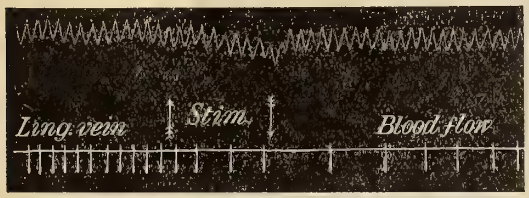

Fig. 1.

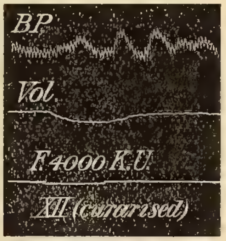

Fig. 2.

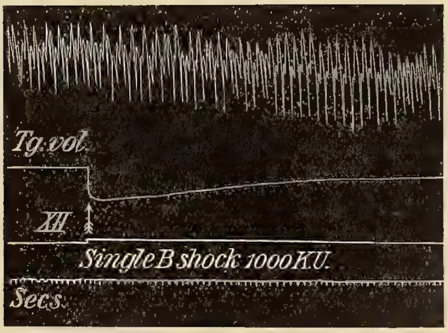

Fig. 3.

Stimulation of the made-up hypoglossal nerve with only a single shock (to avoid any appreciable "metabolite" effect) causes an emptying of the collapsible veins and capillaries by simple compression of the contracting skeletal muscle. It is here suggested that the arterioles, by the simultaneous stimulation of the vaso-constrictor nerves have their lumina rendered less liable to extinction: this would prevent a reversal of the blood stream and maintain a patent system for the onrush of blood following repeated contractions (fig. 4), or a prolonged contraction (fig. 5). It is difficult to imagine what function vaso-constrictors, bound up with a motor nerve, might serve. Fig. 5 shows the steady diminution of the tension developed in the tongue arising from the anæmia produced by its own contraction.

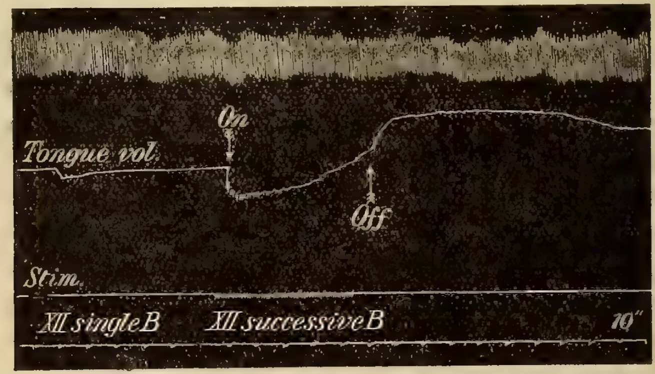

Fig. 4.

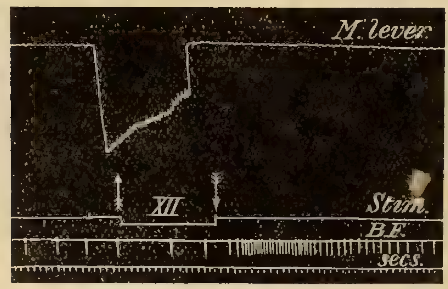

Fig. 5.

4. The Vaso-dilator Nerves.

Fig. 6, in the opinion of the writer, constitutes the most convincing single piece of evidence in favour of the undoubted existence of vaso-dilator nerves. Such a huge vaso-dilation in the absence of any muscular activity must be independent of metabolites. The effect produced by a similar shock upon the motor nerve is seen in fig. 3, where one would expect a greater production of metabolites, yet vaso-dilation is absent.

How very effective a mechanical stimulus is in evoking vaso-dilation (Bayliss, 1920), by excitation of the lingual nerve, is seen in the plethysmographic record of the tongue in fig. 7.

It must be acknowledged, however, that the vaso-dilator fibres have a long latent period (5 seconds), time enough to allow of the production of metabolites. To see the drops of venous blood issuing from the lingual vein suddenly burst into a continuous stream of bright arterial colour on stimulation of the lingual nerve while the tongue remains in a state of restful indifference, is a most simple and convincing demonstration of the individual existence of vaso-dilator nerves.

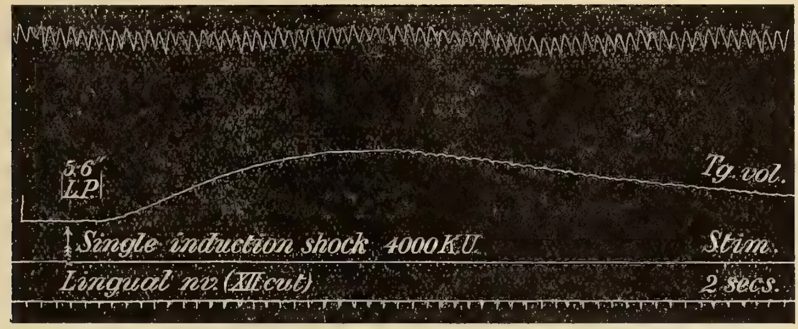

FIG. 6.

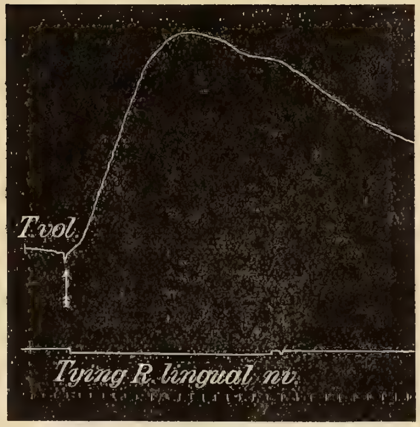

FIG. 7.

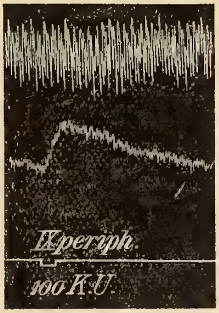

FIG. 8.

Let us examine further the theory which explains vaso-dilation by the action of metabolites. This theory has been based upon the metabolism of the submaxillary gland as measured by its oxygen intake, which is found to be increased in all conditions in which vaso-dilation accompanies excitation of its nerves, and this increased gaseous metabolism only slowly dies down (Barcroft and Kato, 1915). The oxygen consumption in the tongue has been recently measured by Anrep and Evans (1920), and the mean result of their experiments is shown in the following Table.

	Flow of blood.	Oxygen used.
	c.c./min.	c.c./min.
During rest	0·59	0·144
Stimulation of lingual nerve	2·85	0·142

From this it will be seen that the stationary oxygen consumption lends no support to the metabolite theory for the explanation of the five-fold increase of blood flow obtained by these experimenters.

Measurement of the alkali reserve of the blood from the lingual vein and the blood from the femoral artery during stimulation of the lingual nerve shows them to be practically identical. This is seen in the following figures, obtained from different dogs under widely varying conditions:—

Stimulation of lingual nerve.	Alkali reserve of plasma.					
Lingual vein	49·5	53·5	67·0	69	70	48·5
Femoral artery	49·5	54·0	67·5	69	70	49·0

Thus, interrupted faradisation of the lingual nerve produces no measurable difference in the alkali reserve (CO_2 metabolite), even when the vaso-dilation is so great as to increase the blood-flow five to eight times, as in the above experiments (of course, the dilution of the metabolite proceeds at the same rate). In so far as gaseous metabolites are concerned, there is no denying the existence of vaso-dilator nerves.

Furthermore, no real difference was detected in the lactic acid-content of the blood issuing from the tongue during stimulation of the lingual nerve as compared with that in the arterial femoral blood, *e.g.*, femoral artery, 6·4 mgrm. per 100 c.c.; lingual vein, 6·0 mgrm. per 100 c.c.

If vaso-dilator substances are produced when the lingual nerve is excited, then they must necessarily be of a non-oxidative character and presumably of a very powerful nature; the later stages of this investigation will show that such an assumption is unnecessary, since the vaso-dilator nerves of the tongue possess independent functions, and are not subservient to the functional activity of the tongue muscles.

The glossopharyngeal, the sensory nerve of the posterior third of the tongue, responds to stimulation just as the lingual does, but in a milder degree (fig. 8); either it contains specific vaso-dilator fibres (*via* tympanic branch, Loeb and Eckhard), or it is capable of conducting anti-dromic vaso-dilator impulses similar to those found by Bayliss (1901) in the sensory nerves of the limbs.

5. METABOLITES.

Gaskell (1880) was the first to lay stress on the importance of the local vaso-dilator action of acid metabolites; he observed the effect of lactic acid on the curarised mylo-hyoid of the frog, and measured with a micrometer eyepiece the dilatation of the blood-vessels. About the same time Severini put forward the view that "the increased flow of blood through an organ when it is in a condition of activity is due to the trophic dilatation of the capillaries, and not to relaxation of the vascular muscle." He stated that oxygen diminishes the size of the capillary lumen, because the nucleus of the cells of the capillary wall (nucleus of Golubew) becomes more spherical, while conversely with the action of CO_2 it flattens out in the cell, and so the lumen is greater.

More recently, Krogh (1919) has observed the circulation in the thin muscles of frogs and guinea-pigs, chiefly by reflected light, with a binocular microscope; he finds that when muscles contract, either spontaneously or as a result of artificial stimulation, many more capillaries spring into view, and when the contraction is over they disappear again; capillary dilatation also occurs when certain irritants and narcotics are applied locally to the tissue, and in these cases the capillary dilatation is not to be explained by dilatation of the arterioles. This independent state of contractility of the capillaries is seen in the action of histamine, which dilates the capillaries and constricts the arterioles (Dale and Richards, 1918). This drug exerts such a powerful action on the vessels that the injection into an animal of an amount equal to one-millionth of its weight will cause a fall of blood-pressure to one-half and a condition indistinguishable from surgical shock (Dale and Laidlaw, 1919). The possibility of the production in minute quantities of similar substances during cellular activity must be kept in mind, though none have been isolated up to the present. Still, it must be pointed out that the vaso-dilation produced by the action of histamine in cats and dogs is replaced by vaso-constriction in the guinea-pig, so that it would be totally unjustifiable to assert that this substance plays the part of a general metabolite to bring about vascular dilation in active organs. It must also be mentioned that adrenalin in very small doses also possesses a dilator action on the capillaries (an effect which appears to be independent of the sympathetic), but the concurrent action on the arterioles is more pronounced than is the case with histamine (Dale and Richards, 1918).

A similar condition of affairs is seen in chilblains, when the capillaries are distended with blood of venous colour, owing to the impoverished oxygen supply resulting from the simultaneous arteriolar constriction.

Functional Activity.

A plethysmographic record of the tongue during the application of a single induction shock to the hypoglossal nerve (fig. 3) shows a simple compression of the collapsible vessels by the contracting muscles and a complete return to normal without any appreciable after effect of vaso-dilation.

When a tongue is made to develop tension against a muscle lever we note during the application of a series of successive induction shocks (fig. 9) a vaso-dilation which lasts for more than a minute after stimulation (see also Verzar 1912). One of the earlier (imperfect) plethysmographic records shows this hyperæmia very well (fig. 10), the vaso-dilation yielding a pulsatile tongue.

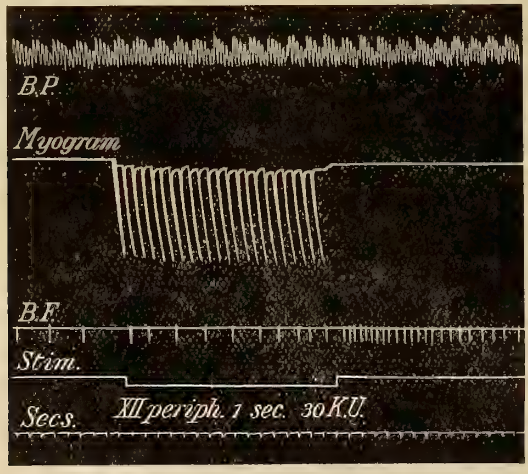

Fig. 9.

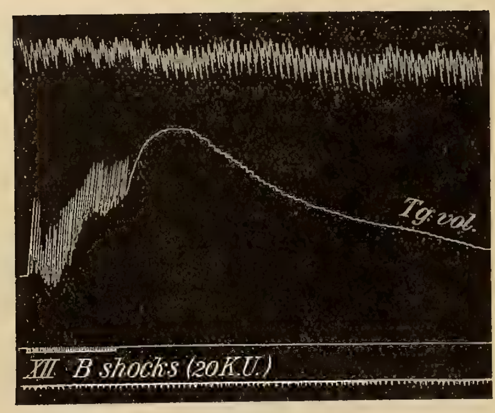

Fig. 10.

During faradisation of the motor nerve the blood flow is almost completely arrested by the compression of the contracting muscles (fig. 11). It is obvious that when a muscle is exerting its maximum effort and there is no alternation of contraction between groups of muscle-fibres, to sustain the effort for a considerable time involves the working of the muscle in the complete absence of fresh blood; in addition to the possible production of metabolites of activity we have here to consider the development of asphyxial products. This is a point of great importance in physical culture, *e.g.*, the maintenance of a continued position like "attention." The phenomenon is seen in its most exaggerated form in muscular "cramp."

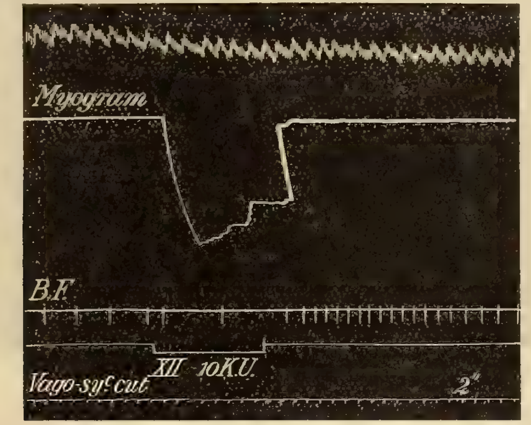

Fig. 11.

Ischæmia: The vaso-dilator effect of asphyxial products may be seen by placing artery forceps on the lingual arteries (fig. 12); on releasing the forceps the tongue dilates to an extent greater than in the resting condition and pulsation becomes more marked. This result confirms the findings of Anrep (1912) for the fore-limb.

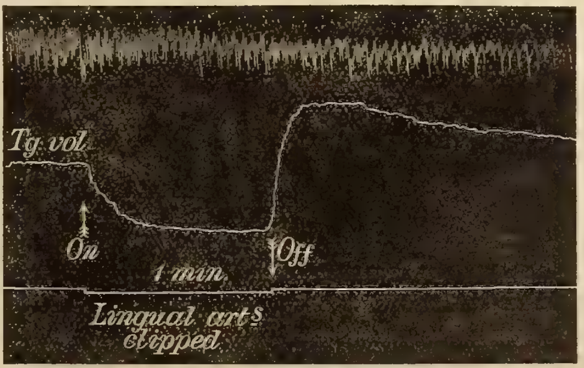

Fig. 12.

Venous Congestion: Compression of the lingual vein leads to venous engorgement of the tongue; on releasing the clip the venules quickly empty but the arterioles and capillaries, probably, remain dilated until the asphyxial products are removed (fig. 13). The long upward curve corresponds to the stage of "passive hyperæmia" while the swelling a few seconds after the release of the compression is a true active hyperæmia; the former is therapeutically regulated in Bier's treatment, though even here the long application of a tourniquet may also involve another doubtful factor—the swelling of the body colloids (M. Fischer, 1920).

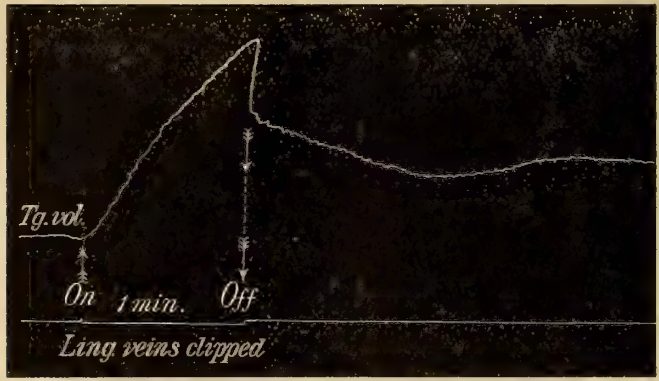

Fig. 13.

Both ischæmia and congestion produce like effects and the asphyxial products arising from them are additive (fig. 14). A corresponding additive effect is obtained with the metabolites of activity and asphyxial products (fig. 15), from which a similarity between these substances may be inferred.

Chemical Vaso-dilator Substances: Schwarz and Lemberger (1911) found that the injection of 1 c.c. of molar HCl into the central end of the left subclavian artery caused obvious dilatation of the blood vessels of the submaxillary gland. Comparing different acids, their action was not found to

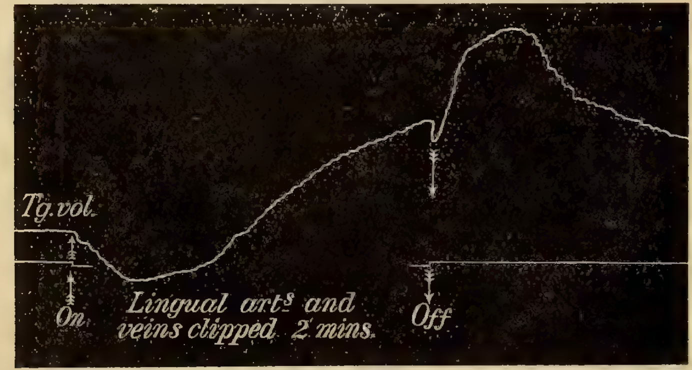

Fig. 14.

correspond to the H-ion concentration. The effect, as they point out, is really produced by CO_2 driven off from the bicarbonate of the blood. Acids weaker than CO_2 were inactive.

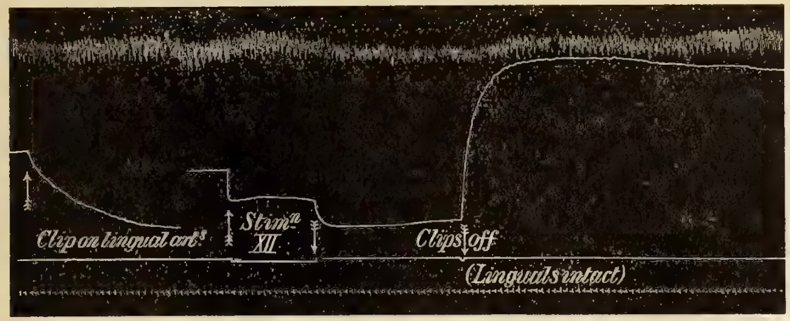

Fig. 15.

In order to put this to a more direct and measurable test, 1 c.c. of Ringer's fluid containing the substance was slowly introduced through a fine hypodermic needle immediately into the lingual artery without occluding it, thus minimising the capacity effect. Plain Ringer, sodium bicarbonate, and lactic acid were found to produce a vaso-dilation in the order named (so that the effect of Ringer alone must be discounted), but the effect produced by lactic acid was much the largest and most prolonged (fig. 16) and was in all respects

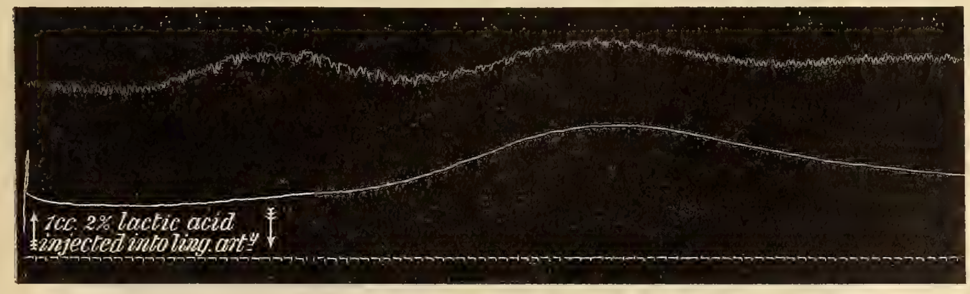

Fig. 16.

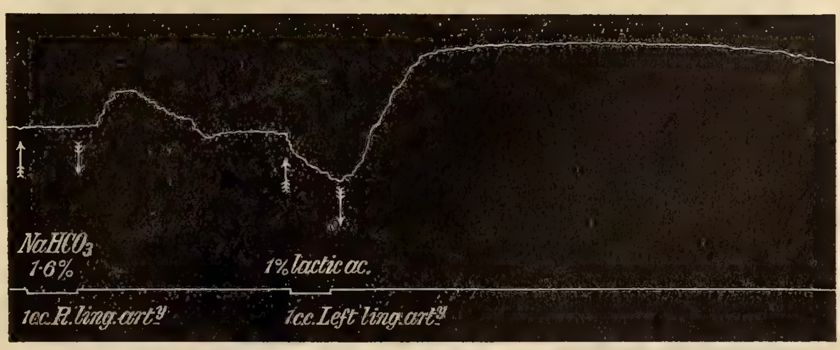

Fig. 17.

similar to the vaso-dilation produced by stimulation of the lingual nerve (compare figs. 17 and 6) and by the normal metabolites arising from muscular activity (compare figs. 17 and 4). Lactic acid, directly or indirectly, is therefore a true vaso-dilator substance.

6. FUNCTIONAL HYPERÆMIA.

We must now arrive at a decision regarding the mechanism by which the vaso-dilation accompanying muscular activity is brought about. Let us consider the first possibility, viz., that some form of reflex excites the vaso-dilator nerves into action at the same time as or soon after the stimulation of the motor nerve.

In glandular organs, the vaso-dilator fibres run in the same bundle as the secreto-motor fibres (*e.g.*, the chorda tympani to the submaxillary gland) and do not admit of direct analysis; the attempt to block one or other by means of drugs is not as convincing as simple section.

In our preparation, however, it is the vaso-constrictor fibres which run with the motor nerve while the fibres with a vaso-dilator action are bound up in the separate sensory nerve; we are thus enabled, by means of section, to interrupt any reflex arc (unless it be a peripheral axon reflex) through which vaso-dilation might be produced during motor activity.

An examination of the accompanying plethysmographic records (figs. 18 and 19) shows that as the initial mechanical stimulation of the lingual nerve (fig. 7) passes off, the vaso-dilation produced by faradisation of the hypoglossal nerve gradually becomes as great after as before section of the lingual nerve.

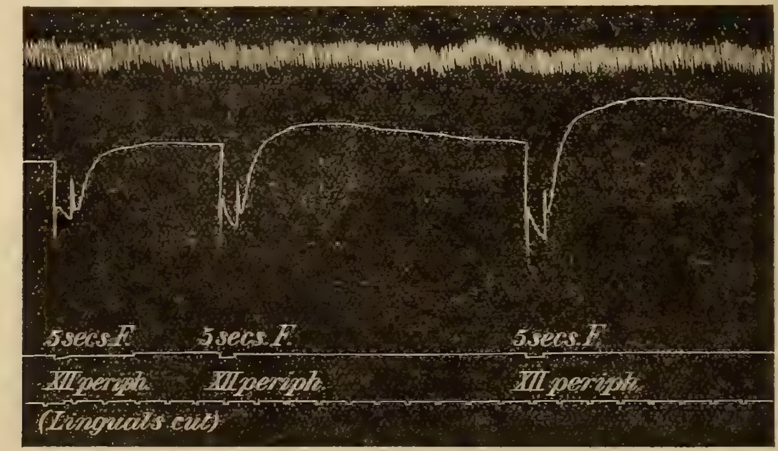

FIG. 18.

Division of the motor nerve makes no difference to the form of the curve, the same degree of dilation being produced by stimulating the uncut nerve as by stimulating the peripheral end of the divided hypo-glossal (figs. 19 and 20).

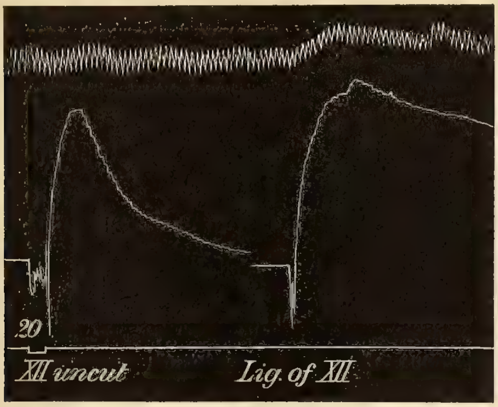

Fig. 19.

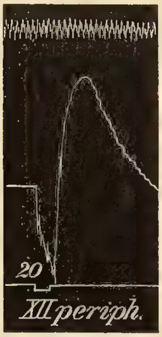

Fig. 20.

A large number of experiments was performed in the attempt to analyse the muscular contractions of the tongue during swallowing, elicited by stimulating the central end of the superior laryngeal nerve. The resulting vaso-dilation was inappreciable, since the long period of rest between successive swallowings (5 seconds or more), allowed of the complete removal of metabolites; and, on the other hand, section of both lingual nerves made no difference.

In the absence of any definite evidence for a reflex excitation of the lingual nerve, we must examine the metabolite hypothesis as a complete explanation for the hyperæmia of muscular activity. We have seen that lactic acid injected into the lingual artery can produce a typical vaso-dilation. Is lactic acid produced in appreciable amount during muscular activity? The answer is in the affirmative, if, in Ryffel's method (1909), it is really lactic acid which is being determined; estimations by this method gave the following results:—

	Lactic acid in mgrm. per 100 c.c. lingual blood during stimulation of	
	Lingual nerve.	Hypoglossal nerve.
Dog 11	6·0	40·8 25·6 (3 mins. later).
„ 12	6·4	53·6 48·0 (1 min. later).
„ 13	16·0*	64·0
„ 14	8·4	40·8
Mean	9·2	49·8

* After resuscitation from temporary asphyxia.

During stimulation of the lingual nerve there is the same concentration within the limits of experimental error, of lactic acid in the blood of the lingual vein as there is in the femoral arterial blood (*vide infra*). Thus one finds the lactic acid increased about five-fold in the venous blood of these experiments, in which the blood flow was maintained at twice its normal rate by interrupted faradisation of the motor nerve; if the normal rate of venous outflow had prevailed then the lactic acid concentration would have risen ten-fold; and in tetanus its concentration must be tremendously high.

The production of lactic acid would lead one to expect a reduction in the alkali-reserve according to the equation

$$H{\cdot}C_3H_5O_3 + NaHCO_3 = NaC_3H_5O_3 + H_2O + CO_2$$

but estimations show a simultaneous increase in the alkali-reserve :—

Alkali reserve of plasma.	Tongue.					
	Resting.		During stimulation of			
			Lingual nerve.		Hypoglossal nerve.	
	Venous.	Arterial.	Venous.	Arterial.	Venous.	Arterial.
Dog 15	55	55	54·5	55	59	54
„ 16	71	70	71	71	82	76
					83	74
„ 17	64	64	—	—	69	64
„ 18	52	50·5	—	—	64	54
„ 19	—	—	49·5	50	57	50
„ 20	61	59	60	59	73	59
„ 21	—	—	47	48	59	43
Mean $v-a$	1		—		9	

This absolute rise of nine in the alkali-reserve on a mean arterial alkali-reserve of 52·5 is equivalent to an average increase of 16 per cent. It is abundantly clear that both CO_2 and lactic acid are important metabolites of muscular activity. Fletcher (1907) found that an evolution of CO_2 follows a production of lactic acid in the frog's gastrocnemius.

The carriage of such large increases of acid by the blood became a problem of so much interest that the relative part played by plasma and corpuscles was investigated. The blood was collected under paraffin, NaF being used as an anti-coagulant, since it possesses the double advantage of not interfering with the estimation, and also preventing glycolysis (Clogne et Richaud), glycolysis having been shown to be associated with the formation of lactic acid in shed blood by Evans (personal communication). The results obtained are shown here :—

Lactic acid (mgrm. in 100 c.c. each of)	Stimulation of			
	Lingual nerve.		Hypoglossal nerve.	
	Plasma.	Corpuscles.	Plasma.	Corpuscles.
Dog 22	16·0	16·0	62·0	68·0
„ 23	12·5	12·9	18·4	16·0
„ 24	8·0	8·8	16·0	20·8
Mean	12·2	12·6	32·1	34·9

Taking into account the hæmatocrite values of these bloods, the lactic acid would appear to be evenly diffused through plasma and corpuscles.

The corpuscles from exercised muscles seemed to show an acidity of their contents markedly higher than the plasma in which they were laked even when NaF was used as the anti-coagulant (*vide infra*). Two dialysing sacs were prepared (Dale and Evans, 1920) and half filled with saline and water respectively (both of known P_H and corrected for) and a comparison made of the P_H; such results as the following were obtained :—

	Dog 25.	Dog 26
Corpuscles intact (in saline)	7·35	7·40
Ruptured corpuscles (in distilled water)	7·20	7·25

It must be mentioned, however, that laked corpuscles (frozen and thawed) always exhibit a slightly lower P_H than intact corpuscles.

An increase of lactic acid in the blood is thus seen to be not incompatible with a rise of alkali reserve in the plasma, in view of the share played by the corpuscles in bearing the onslaught of the acid attack. It must further be admitted that the red corpuscle is permeable to the lactic acid ion, and the following is advanced as a possible explanation of the events :—

1°. Diffusion of the lactic acid from the working muscle into the alkaline tissue fluid

$$\text{Lactic acid} + NaHCO_3 \longrightarrow \text{Sod. lactate} + H_2CO_3.$$

The carbonic acid given off diffuses through the vessel wall where it exerts its vaso-dilator effect (*vide* figs. 4 and 17; note in the latter the direct astringent effect and the indirect effect of lactic acid due to CO_2).

2°. Diminution of the plasma chlorides and increase of the alkali reserve (L. J. Henderson, 1909)

$$\underset{\text{from } 1^\circ}{H_2CO_3} + \underset{\text{of plasma}}{NaCl} \rightleftharpoons \underset{\text{alkali reserve.}}{NaHCO_3} + HCl.$$

3°. Some free lactic acid is formed from part of the sodium lactate in (1) and part of the HCl in 2°

$$\text{Sod. lactate} + HCl \longrightarrow \text{Lactic acid} + NaCl.$$

This lactic acid penetrates the corpuscles just as the HCl from 2° does (Hamburger, 1904) and the H_2CO_3 from 1° (Buckmaster, 1918).

4°. The oxidative removal of lactic acid continues for some minutes (Fletcher and Hopkins) and coincides with the recovery heat production (Hill) and the increased oxygen intake (Verzàr) following muscular activity.

It is believed by many, though on insufficient data, that lactic acid and CO_2 act by increasing the H-ion concentration. Calculation of the P_H of the blood from measurements of the alveolar CO_2 may show a difference of 0·02 during muscular exercise (Campbell, Douglas, Hobson, 1914); a rise of acidity has been inferred from estimations of the affinity of the blood for oxygen (Mathison); more recently Barcroft and Parsons (1920) have found that muscular work causes a fall in P_H of 0·08 in the blood when defibrinated and completely reduced.

The erythema arising in electro-therapeutics in the area of the kathode suggests the possibility of a direct action by the H-ions (or Na-ions ?). In normal activity, such a direct action of lactic acid is impossible in the presence of the bicarbonate of the tissue fluids; the increase of alkali reserve seen to occur above must also tend to keep down the H-ion concentration of the *plasma*, hence the ultimate depressant of the blood-vessel wall must be the CO_2. Patterson and Starling (1914), working on the isolated heart-lung preparation, have shown that the heart relaxes more and more as CO_2 is added to the air ventilating the lungs. Evidence is accumulating that CO_2 possesses specific properties in its action upon the tissues which are not possessed by other acids (Schwarz and Lemberger, 1914, C. Lovatt-Evans); fig. 17 is interesting in this connection.

Finally, we have shown in § 5 that CO_2 and lactic acid, when injected into the blood stream, act as powerful vaso-dilator substances, and again in § 6 that these acid metabolites are formed in increased amount during muscular activity. In so far as functional hyperæmia is adequately explained by the vaso-dilator action of metabolites, we are in full agreement with Barcroft (1914); but in § 4 we have shown the separate existence of vaso-dilator nerves which were not, at least in the above experiments, called into play during muscular activity. We must consequently expect to find other functions for the vaso-dilator nerves.

7. Control of Body Temperature.

The fortunate employment of the dog gave the clue to a possible function of the vaso-dilator fibres in the lingual nerve. It is a common fact that dogs resort to panting and extrusion of the tongue for cooling of the blood. The effect upon the blood-flow in the tongue resulting from the stimulation of the heat receptors in the skin was therefore studied.

The fur was cut off, a radiant-heat hemi-cylinder was placed over the trunk of the animal, the venous outflow from the lingual vein recorded, and the rectal temperature observed throughout the experiment.

The warming of the skin caused hyperæmia and increased lingual blood-flow long before the body temperature was appreciably raised (fig. 21, i and ii). In all cases where this response did not occur the body temperature steadily advanced; Stewart (1913) also emphasises the importance of reduced blood-flow in fever.

To test whether this was an effect of heated blood directly upon the vessels or a reflex mechanism, the lingual nerves were cut on both sides and time given for the effect of this stimulus (fig. 7) to pass off. The application of radiant heat to the skin of the trunk was now not followed by lingual hyperæmia (fig. 21, iii and iv).

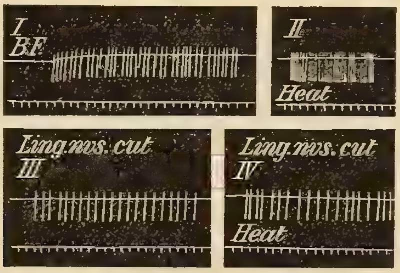

Fig. 21.

The same results were obtained in another dog, which was immersed in a warm bath. It is thus apparent that a nervous reflex of this nature brings about a quicker response than could be obtained by the heating up of the whole blood; and, further, the nervous reflex ensures vaso-dilation of that particular organ which is most favourably disposed for the cooling of the blood, whereas heated blood would have a generalised effect on external and internal organs alike. It is possible that the flushing of the face in man in emotional states and in fever may also be accounted for by similar reflexes involving vaso-dilator nerves. Measurements of the blood-flow, by Stewart's method, in the hands and feet during fever show that the cutaneous vessels dilate during fall of temperature by *crisis*.

Whether the vaso-dilation of the tongue plays any part in the sensation of taste was not investigated.

CONCLUSIONS.

1. The lingual nerve contains true vaso-dilator fibres, just as the sympathetic contains vaso-constrictor fibres; both are equally independent of the intervention of metabolites.

2. No evidence was found that functional hyperæmia is due either to diminution in vaso-constrictor tone or to increase in vaso-dilator tone.

3. The experiments show that the increased blood-supply during muscular activity is due entirely to the products of metabolism; the absence of a simultaneous excitation of the vaso-dilator nerves during voluntary movement, though probable, is not proved.

4. Of the metabolites estimated, CO_2 and α-OH organic acids were found to be increased.

5. Apart from muscular activity, one function of vaso-dilator nerves was found to be concerned with the control of body temperature; active hyperæmia in the dog's tongue may be induced by reflex excitation of the vaso-dilator nerves through stimulation of heat receptors in the skin.*

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* *Note.*—The expenses of this work were defrayed by a grant from the Government Grant Committee of the Royal Society.

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The Acidity of Muscle during Maintained Contraction.

By H. E. Roaf.

(Communicated by Sir Charles S. Sherrington, Pres. R.S. Received March 11, 1922.)

(From the Department of Physiology, London Hospital, Medical College.)

In 1913, I described a method for recording changes in hydrogen-ion concentrations in tissues, by means of a manganese dioxide electrode in combination with a calomel electrode (1). By this method it was shown that the acidity of muscle probably increased at the same time as, or slightly before, the tension increased, and that the acidity decreased as the muscle relaxed (2).

In a paper, which appeared as this note was being prepared for publication, Ritchie* states that he has been unable to detect a variation in acidity by the use of manganese dioxide electrodes. I am inclined to think that his failure is due to the injury to the muscles on insertion of wires into its substance. In my own experiments the wires rest on the surface of the muscle.

The electrical change observed by me is not due to spread of potential from the stimulating circuit, because in some records a potential is recorded corresponding to the time of stimulation. This potential is quite separate from the larger potential ascribed to the acid production, and it may be in the same or in an opposite direction from that which accompanies the contraction.

In my experiments I took precautions to minimise movement of the electrode on the surface of the muscle, and any slight movements are probably less important with the sartorius than with the gastrocnemius. Change of potential, due to movement of the electrode, would probably vary in direction, whilst with the manganese dioxide electrode the change is always in the same direction. If the electrode did move on the surface of the muscle it is unlikely that the potential would return to its former value, as it is seen to do in most of my records.

Other forms of metallic electrodes would give changes of potential depending on the chemical reactions that take place in contact with them.

I do not think that the change shown by a manganese dioxide electrode is due to polarisation. If the electrodes are so placed that no difference of potential is shown during contraction by non-polarisable electrodes, it is difficult to see how any difference of potential will be produced when one calomel electrode is substituted by a manganese dioxide electrode and the contact potential has been properly balanced. If there is no difference of potential there cannot be any current, therefore there will be no polarisation.

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* A. D. Ritchie, 'Journ. Physiol.,' vol. 56, p. 53 (1922).

If acidity is related to tension in muscle it is necessary to show that acidity remains when the muscle tension is maintained, and decreases when the tension falls. In order to test the relation of acidity to muscle tension two experimental procedures were tried.

In the first, veratrin was painted on the frog's muscle after a record of the normal contraction had been made. The out-standing feature of the action of veratrin is that it does not affect the increase in tension of the muscle, but that it delays the relaxation.

With such a preparation the acidity, as shown by the manganese dioxide electrode, remains also. Fig. 1 shows a muscle twitch of a fresh sartorius, whilst fig. 2 shows the result with the same muscle after it had been treated with veratrin ($1 : 10^5$).

For the second procedure a decerebrate cat was used. This preparation shows a marked tension (decerebrate rigidity) in the limb muscles. The tension can be abolished by cutting the efferent nerves to the muscles, or by reflex inhibition as the result of stimulation of an afferent nerve.

In decerebrate preparations the rigid muscles show a greater acidity than when the muscles are paralysed by cutting their efferent nerve supply, as shown by the following results:—

Table I.—Decerebrate Preparations showing Potential in Volts between a Manganese Dioxide Electrode and a Calomel Electrode.

Date.	*R. Sartorius.*			*L. Sartorius.*		
	Rigid.	After cutting nerve.	Difference.	Rigid.	After cutting nerve.	Difference.
21.7.13 ...	0·325	0·004	0·321	0·186	−0·169	0·355
14.7.14 ...	0·312	0·249	0·063	0·249	0·242	0·007
8.3.21 ...	0·355	0·206	0·149	0·866	0·771	0·095
		Average	0·178		Average	0·152

Although the absolute values of these are unreliable it is clear that paralysing the muscle always gives a result corresponding to a decrease in acidity.

In order to demonstrate further that removal of maintained contraction causes decrease in acidity, a reflex vasto-crureus preparation was used (3). The sartorius was removed and the electrodes placed on the surface of the vasto-crureus. On causing a reflex inhibition by stimulation of the ipsilateral

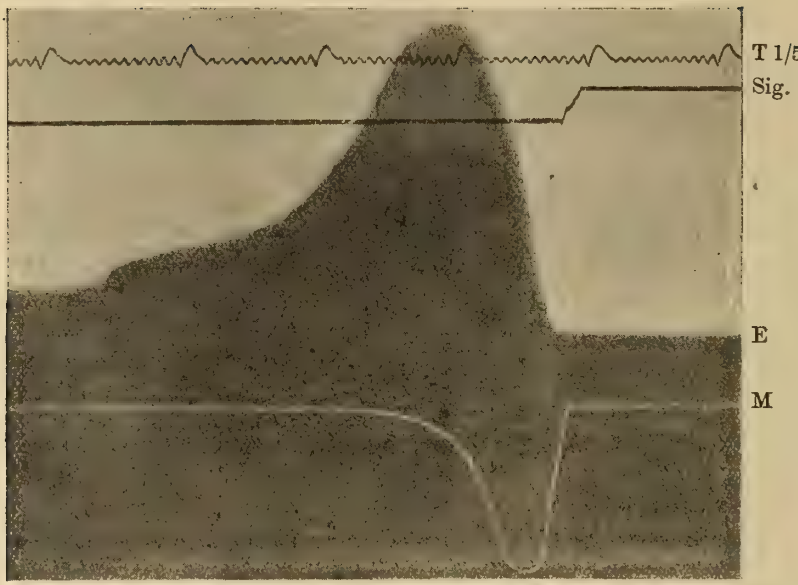

Fig. 1.—Simple muscle twitch.

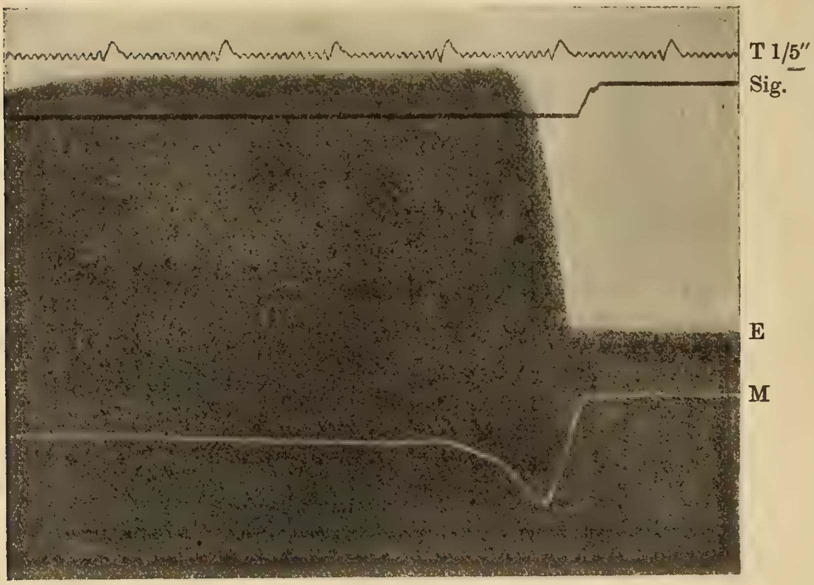

Fig. 2.—Record of same muscle after treatment with veratrin (1 : 10^5). Records read from right to left. Top line, time in 1/5″. Second line, signal for single break-shock given to muscle. Bottom line, downward movement shows increased tension by muscle. Shadow shows movement of mercury in capillary. Movement upwards corresponds to increased acidity at manganese dioxide electrode.

sciatic nerve, as shown in fig. 3, the end result corresponds to a decrease in acidity. The initial result is still doubtful, as the figure reproduced shows a movement of the mercury in the opposite direction before the inhibition occurs. This result may be due to either a slight contraction before relaxation,* or setting free of acid from the muscle preliminary to its removal by some other mechanism.

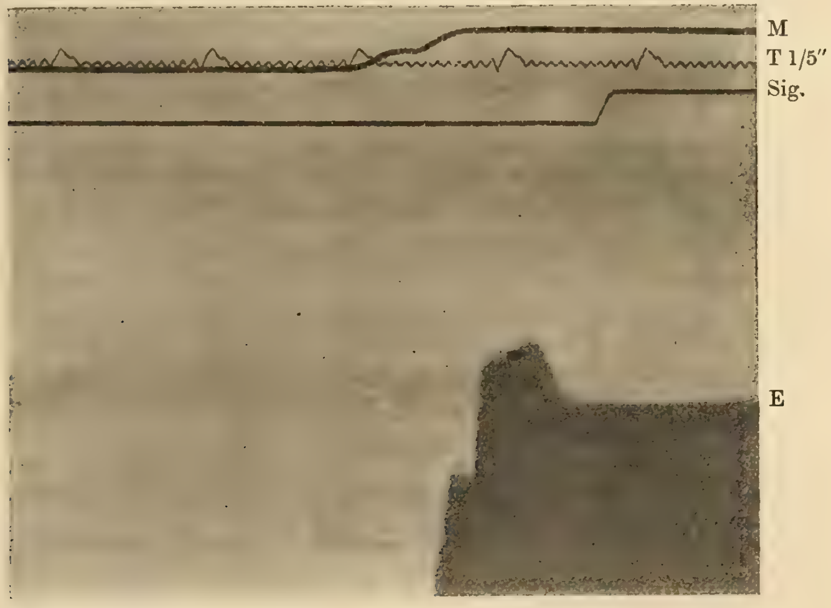

Fig. 3.—Record of reflex inhibition. Indications as in figs. 1 and 2, but relaxation of muscle is shown by a line which starts above time marker and falls across it, instead of the record of contraction shown below. Relaxation is accompanied by decrease in acidity.

The results indicate that acidity and tension in muscle are concurrent. Thus it may be better to investigate, not how the contraction is maintained, but why the acid remains and is not removed as in a simple twitch. The results further suggest that as acidity is common to both tetanus and tone probably the mechanism for the production of both is the same, *i.e.*, that there is one mechanism in muscle and not two.

I wish to thank Mr. F. C. Smith for assistance in some of the experiments on decerebrate cats. Some of the apparatus for the research was obtained

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* Sir Charles Sherrington informs me that weak reflex inhibition is frequently preceded by a slight contraction. This fact is in favour of the view that the slight increase in acidity, shown before relaxation occurs, is due to a preliminary increase in tension.

by grants from the Government Grant Committee of the Royal Society, and from the London Hospital Medical College Research Fund, and part of it was given by Mr. H. S. Souttar.

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On the Heat Production and Oxidation Processes of the Echinoderm Egg during Fertilisation and Early Development.

By C. Shearer, F.R.S.

(Received March 25, 1922.)

(From the Biochemical Laboratory, Cambridge.)

Introduction.

In the following experiments an attempt is made to measure the heat liberation of the ovum on fertilisation and early development, and to correlate this with the amount of oxygen consumed and the carbon dioxide given off at the same time. New methods hitherto unused for this purpose have been employed. The question has already been investigated by Meyerhof (1) in an extensive paper published in 1911. He determined the heat production and the oxygen consumption of the egg of the sea-urchin *Strongylocentrotus* on fertilisation and early development. The heat production was measured directly by means of a finely divided Beckmann thermometer, while the eggs were contained in a small closed vacuum flask completely submerged in the water of a carefully regulated thermostat. The oxygen consumption of the eggs was at the same time determined at intervals of an hour, by the titration of the sea-water in which the eggs were kept with sodium thiosulphate by the Winkler method.

The heat given off by a known quantity of eggs expressed in gram calories per hour, divided by the amount of oxygen consumed in the same time expressed in milligrams, gave him a calorific quotient which he calls "Q." This he found for the early stages of segmentation to be about 2·75, but if the heat of solution of carbon dioxide to form bicarbonate with the sea-water is taken into consideration this value becomes 2·6. This figure is so low, however, as to suggest that his data for the heat liberation or the oxygen consumption are incorrect, or that the oxidation processes of the egg-cell on fertilisation are of a different character from those of adult metabolism. It has been shown by Zunst and Schumberg, Rauber, Pflüger and others, that when fat is consumed this figure should be in the vicinity of 3·3, when protein 3·2, and carbohydrate 2·9. Meyerhof could find no carbohydrate in the egg, and there could be no destruction of protein, but sufficient fat was found in the egg to give the quotient observed. In the case of fresh sperm, Q was 3·1 or nearly normal. The carbon dioxide production by the eggs was not measured.

The most important fact, however, arising from Meyerhof's experiments was that, whether he took the unfertilised egg, the fertilised, or the fertilised egg treated with phenylurethane, so that cell formation was inhibited although development proceeded, he found the value of this calorific quotient was always the same. If any of the chemical energy liberated in the egg as the result of the increased oxygen consumption of the egg on fertilization were utilised in producing the visible morphological structure of the egg, then the value of this quotient could not be the same in all these instances. Warburg (2) had already pointed out, that the oxygen consumption of the egg-cell on development always fails to keep pace with the increase in morphological structure. In *Arbacia* he found the fertilised egg in the one cell stage during the first hour of development consumed 4 c.c. of oxygen; in the sixth hour, the same quantity of eggs consumed only 6·8 c.c., although now the eggs were each composed of thirty-two cells instead of one.

In another experiment where a larger number of eggs were employed, 13·2 mgrm. of oxygen was consumed by the eggs in the eight-cell stage, while in the thirty-two cell stage only 20·5 mgrm. was absorbed. Thus, while the oxygen consumption doubled in amount the cellular structure had increased four-fold.

Meyerhof found the heat production of a quantity of unfertilised eggs containing 140 mgrm. of nitrogen (about 17 million eggs) to be about 0·9 grm. calories per hour, while the same quantity of fertilised eggs, liberated 4—4·2 grm. calories in this time. In the second hour, the two-cell stage, the heat production rose to 4·5–5 grm.-calories. In the fourth hour, corresponding to the 8-cell stage, it was 6–6·5 grm.-calories. In the sixth hour, the thirty-two-cell stage, it was 9·8 grm.-calories, and from this time onwards the heat liberation increased rapidly, until in the eighteenth hour, when the free swimming stage was reached, it was 17·8 grm.-calories per hour, or four times what it was in the first hour of development. Once development was initiated the heat production rose steadily without pause or interruption. It followed the oxygen consumption closely in all respects, and like this, showed no direct relationship to the rate at which morphological organisation took place within the egg. No heat production could be observed during the formation of the fertilisation membrane or the early phases of the fertilisation process itself.

In all Meyerhof's experiments great over-crowding of the eggs necessarily took place, and it is difficult to believe that under such conditions the heat production was normal. In attempting to repeat his experiments, using a much larger vacuum flask and a smaller quantity of sea-urchin eggs where they were less crowded, I was unable to get them to fertilise in the closed flasks. As Loeb first pointed out, an abundant oxygen supply is the invariable constant required by the fertilised and developing egg-cell. In my own experiments, in order to get my eggs to fertilise and segment regularly, I was forced to adopt some means of keeping them aërated during the course of the experiment. If large quantities of eggs were employed (300–400 mgrm. of egg nitrogen), then it was absolutely necessary to carry out artificial aëration, or otherwise a large number of the eggs quickly died and soon cytolysed, and during cytolysis liberated an abnormal amount of heat. As I have shown with bacteria (3), the death process and cytolysis of all cells is probably accompanied by an abnormally high oxygen consumption and heat liberation. On these grounds Meyerhof's experiments seemed open to criticism. It was worth while repeating his experiments, using different methods which avoided, as far as possible, this difficulty. Moreover, it was of interest to determine if a different method of measuring the heat liberation would give figures similar or of the same order as those obtained by Meyerhof.

The Winkler titration method employed by Meyerhof in estimating the oxygen consumption of the egg on fertilisation and development is somewhat unsatisfactory in that it probably gives too high a figure for the oxygen consumption of the egg. The sea-urchin egg on fertilisation discharges a certain amount of organic slimy material into the sea-water, which interferes to a considerable extent with the accuracy of the titrations carried out by this method. The following experiments are for these reasons, to a large extent, a repetition of Meyerhof's work, using different methods for both the heat measurement and the oxygen consumption and carbon dioxide output of the egg. The eggs and sperm of *Echinus miliaris* were employed. This species being a shore form it is exceptionally favourable for work of this kind. It can be readily reared to the adult stage in small culture jars under laboratory conditions. I have shown, working in conjunction with

De Morgan and Fuchs (4), that this species can be readily raised to the sexually mature F_2 generation in the laboratory if a few simple rules are followed in rearing the larvæ.

Methods.

In making the heat measurements the differential calorimetric method has been adopted instead of the direct method employed by Meyerhof. It requires no expensive fittings or elaborate thermostats, and has the advantage that a number of separate determinations can be made at the same time. All the following experiments were carried out so that the eggs were efficiently aërated. This was carried out so as not to interfere with the accuracy of the heat estimations. To test this point many preliminary experiments were made.* All final calibrations were carried out under conditions identical with those of an actual experiment; the mean of 30 or 40 determinations being taken as the final figure.

The oxygen and carbon dioxide determinations were carried out by the employment of a special pattern of the Barcroft (5) differential manometer, in which it was possible to fertilise the eggs in the closed chamber of the instrument. It was thus possible to record the oxygen consumption and carbon dioxide output of the eggs while the sperm were actually making their way into the egg. As this instrument and the mode of its use has already been described in a previous paper (6), it is unnecessary to give an account of it here.

In the heat measurements, the form of differential calorimetric method employed has been that devised by A. V. Hill (7), and has already been clearly described by him at some length. The method is based on the fact that, within fairly wide limits, a vacuum flask may be given any desired rate of conduction of heat to the outside by simply increasing or decreasing the volume of its fluid contents. By placing the right quantity of fluid, in this case eggs in sea-water, in one flask, and an appropriate quantity of plain sea-water in another flask acting as a control, the two flasks can be given the same temperature fall. They can then be used in making a differential determination, on being connected with one another by means of a thermocouple, with one junction in each flask. The thermocouple being in circuit with a delicate galvanometer, any deflection of the mirror gives the difference of temperature between the two flasks. A copper-constantan thermocouple was used in circuit with a sensitive Ayrton-Mather galvanometer. The sensitivity of the galvanometer was such that, at 3·5 metres distance, every millimetre of the galvanometer scale represented 0·00139° C. The leads from the galvanometer and thermocouples were brought to a specially constructed dial box furnished with two keys, by which three or four thermocouples could be thrown into circuit with the galvanometer, and also small resistances introduced in any of these circuits as desired. All leads and terminals, including those of the galvanometer, were made of copper throughout, thus avoiding any possible thermo-electric effects.

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* Aëration was carried out by bubbling a very small volume of water-saturated air simultaneously through both flasks at regular intervals, the eggs in the flask being previously well aërated for 20 minutes before the commencement of the experiment.

The vacuum flasks were the ordinary narrow-necked silvered Dewar flasks, made as " refills " for commercial thermos bottles. They were used in two sizes, having a capacity of 400 c.c. and 800 c.c. respectively. The larger size have a coefficient of heat loss half that of the smaller, and are therefore more accurate to work with where sufficient experimental material can be obtained. The selection of the flasks was carried out in the following manner :—Some 40 to 50 flasks were obtained for rough testing. These were all filled with the same quantity of water at 60° C. They were then closed with plugs of cotton wool, and put aside in a corner of the room free from draughts, and allowed to warm up for an hour. Their temperature was then taken with a Beckmann thermometer, after which they were allowed to stand for 24 hours, when their temperature was again taken with the Beckmann thermometer. It was usual to find four or five flasks out of the lot that had very similar rates of temperature fall, and these were selected for further calibration. Their coefficients of heat loss were then carefully worked out, under conditions as similar as possible to those obtaining in experiments by the use of the formula $T - T_0/A - T_0 e - kt$, a mean of ten or twelve determinations being taken. The final calibration was carried out under actual conditions of an experiment, with air bubbling through the flask contents, and air tubes and thermocouple junctions in position, and the flask itself sunk down in the water of the thermostat. One flask was given a slightly higher temperature than the other, which was the exact temperature of the bath water; as the temperature of the flask under calibration fell slowly to that of the control flask, a series of readings were taken with the thermocouple and galvanometer; these, on being plotted out, gave a curve from which the value of k could be directly taken. The value given by this last method was the one actually employed for experiments. As a matter of fact, both methods gave very similar values for k in nearly all instances. Prof. Hill was kind enough to place at my service two flasks of 400 c.c. capacity, which were remarkable for having almost the same value of k. They could be used differentially by placing the same amount of fluid in each. They have been extensively used in the present experiments.

The flasks during the course of work were recalibrated from time to time, and at long intervals were tested by the liberation of a known amount of heat in each flask from a small coil of constantan wire. This coil liberated 21 grm.-calories of heat per hour in the flask under the conditions of the test, and the galvanometer scale readings were usually within 3 per cent. of this value. To close the mouths of the flasks during an experiment, it was found that thick wads of cotton wool were the most effective. When the flasks were closed by rubber stoppers and the flask sunk completely in the water of the thermostat, it was found that more heat was lost by conduction through the stopper than was the case when they were only plugged with cotton wool and sunk up to their necks in the water of the bath. The flasks were mounted in pairs in open wirework baskets, which were made so that they could be clamped on the thermostat, so the flasks were held firmly submerged up to within a centimetre of the tops of their necks in the water of the bath. The thermostat tank held 50 or 60 litres of water, and was kept stirred and in uniform temperature throughout, by having compressed air bubbled through it from a number of jets distributed evenly over the bottom of the tank. This method of stirring was very effective, for, when it was in action, it was seldom possible to distinguish more than a hundredth of a degree C. between any two points in the water of the tank. The sides of the tank were protected externally by thick layers of felt, and its inner side was surrounded by a coil of piping through which cold water could be circulated, and the temperature of the tank kept constantly at 14·5° C. The room in which the experiments were conducted was almost entirely underground, and underwent little change of temperature between day and night, or from one day to another, if the door was kept closed and the windows protected. The experiments were carried out in the months of July, August and September, when weather conditions were most favourable for work of this kind. It is the special merit of the differential method that external temperature conditions can be largely neglected, so long as both flasks used in making the differential determinations are affected to the same extent by all variations of external temperature.

In order to get the eggs to fertilise and segment regularly in the flasks, it was found necessary to carry out some form of aëration. To accomplish this, air was slowly bubbled through the contents of both flasks at regular intervals during an experiment. This also served to stir up the eggs and prevent their settling in a dense mass in the bottom of the flasks. The air used in the aëration and stirring process was first passed through a large wash-bottle, half-filled with sea-water, sunk in the middle of the thermostat tank. The air from this bottle was then led into each flask by fine rubber tubes, which passed through the cotton plugs used to close the flasks. The air thus saturated with moisture and at the same time brought to the temperature of the water bath, as Hill has shown, the heat capacity of air being so low, produces little or no cooling effect on the contents of the flasks. At the commencement of an experiment care was taken to adjust both flasks, but especially the control flask, to exactly the same temperature as the thermostat water, which, as already mentioned, was kept constantly at 14·5° C. This adjustment in the case of the control flask was always made to within a hundredth of a degree C. with a Beckmann thermometer. This adjustment was usually carried out several times in succession before an experiment was actually commenced. The sperm were suspended in a small bottle in the water of the thermostat, so that when finally added to the eggs in the flask they were at approximately the same temperature.

On the addition of the sperm the cotton plugs, with the thermocouple junctions and air tubes, were immediately replaced in the necks of the flasks, and galvanometer readings commenced. Readings were always taken at fairly frequent intervals at the commencement of an experiment, but once the experiment was under way, they were usually taken at intervals of several hours. The readings obtained, in millimetres on the galvanometer scale, were then plotted out on squared paper with respect to time, and a curve of observed heat production obtained. As the flasks are meanwhile losing heat, a correction for heat loss has to be made. The loss of one flask, however, by the conditions of the experiment has been made the same as that of the other, so that the rate of temperature-rise in the flask containing the eggs is immediately given by the formula $k(T-T_1)$, where k is the coefficient of temperature-loss of the flask, and $(T-T_1)$ is the difference of temperature between the two flasks, as shown by galvanometer scale readings in millimetres at any instant. The total temperature-rise in the flask is obtained by integrating $k(T-T_1)$ with respect to time, and this value is accurately given by measuring the area of the curve given by the galvanometer scale readings plotted against time. The total heat produced is equal to the capacity of the flasks and fluid multiplied by the final temperature difference between the flasks plus this value of k (times area of curve), where this last expression is equivalent to k [value of the middle ordinate of $(T-T_1)$].

In the following sections of the paper, a few only of the many experiments carried out have been described. In many instances the eggs or sperm, for one reason or another, were unsatisfactory and the experiment failed to give a result. In other experiments, while the eggs and sperm were perfectly ripe and the eggs gave a very high percentage of fertilisation, they failed to fertilise in the manometers to the same extent as they did in the flasks, so rendering comparison of the oxygen intake with the heat production impossible. It is essential in experiments of this kind that in both the oxygen and CO_2, and also the heat determinations, all the eggs should fertilise at the same time, and that, after fertilisation, they should all develop at the same rate, as otherwise no comparisons can be made between different portions of the experiment. In all, out of some 500 experiments few were satisfactory in all respects.

To simplify matters the manometer readings in the following experiments have all been reduced to standard barometric (760 mm. Hg.) pressure and uniform temperature of 14·5° C. The galvanometer scale readings have also been adjusted to start from zero, although it was seldom possible to adjust the temperature of the two flasks so closely that the readings should actually commence at zero. The galvanometer mirror deflection being always either to the right or left the zero was in the middle of the screen. The thermocouple was arranged so that the hottest junction should always deflect the mirror to the right.

Experiment 1.

(a) *Heat Determination.*—400 c.c. of ripe well washed *E. miliaris* eggs in sea-water were placed in Flask R, and 380 c.c. sea-water in Flask No. 7, under the conditions of the experiment both flasks had the same coefficient of heat loss. The flasks were sunk down in the bath water, with the thermocouple junctions and air tubes in place. The temperature of the flasks was adjusted to within a hundredth of a degree of the temperature of the bath, and the flasks allowed to remain with air bubbling through them for half-an-hour. The flasks were closed with thick wads of cotton-wool. At the end of this time the temperature of the flasks was again adjusted as near as possible to that of the bath, and after a few minutes a few drops of sperm were added to the eggs, and the cotton plugs with air tubes and thermocouple junctions replaced in the flasks and galvanometer readings commenced.

Time 3 P.M. galvanometer scale reading was 0
„ 4 „ „ „ 5 mm. to R containing eggs.
„ 5 „ „ „ 7 „ „ „ „
„ 6 „ „ „ 10 „ „ „ „
„ 11 „ „ „ 21 „ „ „ „
„ 2 A.M.* „ „ 28 „ „ „ „

At the end of the experiment 98–100 per cent. of the eggs in the flask were in normal morula stages. Kjeldahl determination gave 58·4 mgrm. egg nitrogen present. In the first hour after the addition of the sperm the eggs liberated 2·9 grm.-calories, 10·5 grm.-calories in the fifth hour, and 22·8 grm.-calories in 11 hours (see Curve, fig. 1, *a*).

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* In carrying out an experiment the preliminary preparations often took up so much time that the actual galvanometer readings could only be started late in the day and had sometimes to be carried through to the following morning.

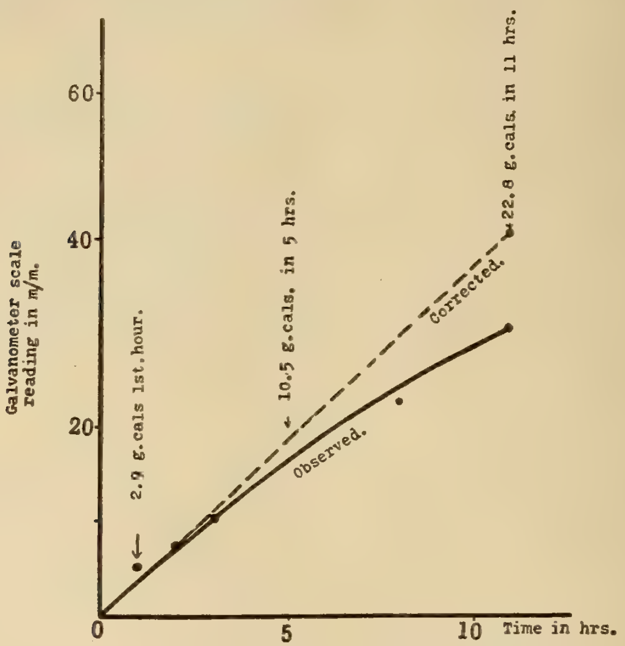

FIG. 1, *a*.—Curve of heat production for Experiment I. 58·4 mgrm. egg nitrogen.

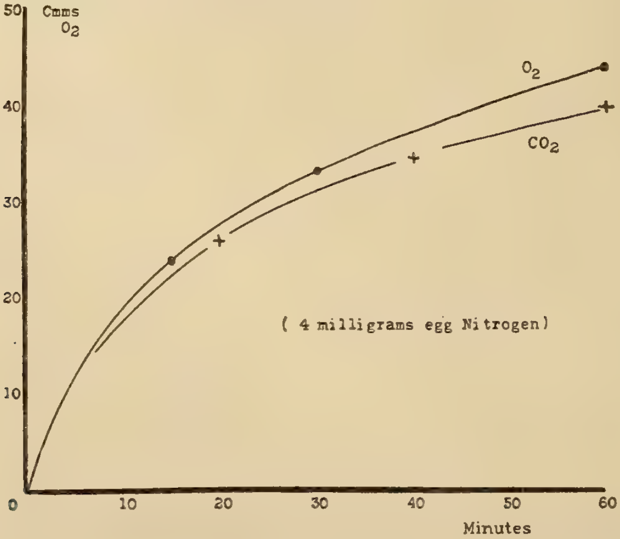

FIG. 1, *b*.—Curve of O_2 consumption and CO_2 liberation for same Experiment I. 4·07 mgrm. egg nitrogen.

(b) *Oxygen and Carbon Dioxide Determination.*—2 c.c. of the same lot of eggs as those placed in the flask in above experiment were put in the chamber of oxygen manometer and a drop of KOH put in the cup, 2 c.c. of plain sea-water being placed in the control chamber. In a second manometer used for control the same quantity of eggs were placed in the chamber, the KOH being left out.

Temperature of manometer bath 14·5° C., barometer 760 mm. The manometers were put in position in the bath and after being brought into complete equilibrium with the bath water the cocks of the manometers closed and the eggs fertilized. The first manometer showed that:—

At the end of 15 minutes the eggs had consumed 24·2 c.mm. oxygen.
" " 30 " " " 33·0 " "
" " 60 " " " 44·0 " "

At the end of the experiment eggs in the chamber showed 100 per cent. fertilisation membranes and some commencing two cell stages. Kjeldahl determination on the eggs gave 4·07 mgrm. egg nitrogen present. 58·4 mgrm. of egg nitrogen at this rate would take up 631·4 c.mm. oxygen, which is equivalent to 0·902 mgrm.

As 2·9 grm.-calories of heat were given off in the first hour following fertilisation, for 58·4 mgrm. N in first part of experiment,

$$Q = \frac{2{\cdot}9}{0{\cdot}902} = 3{\cdot}215.$$

The heat production in this experiment was 10·5 grm.-calories in 5 hours; in 12 hours, 22·8 grm.-calories.

In the second manometer 4·0 c.mm. of oxygen seemed to be the difference between the oxygen intake and CO_2 output in this experiment, for 4·07 mgrm. of egg nitrogen. If we assume that 44·0 − 4·0 gives us the amount of carbon dioxide produced in this experiment, we get the respiratory quotient of 40/44 = 0·91.

It will thus be seen the carbon dioxide output of the eggs is almost as great as the oxygen consumption. In all the experiments the carbon dioxide respiration follows the oxygen consumption very closely, the respiratory quotient varying from 0·9 to 0·95 in different experiments.

Experiment 2.

(a) *Heat Determination.*—800 c.c. of well washed ripe *E. miliaris* eggs in sea-water placed in Flask No. 3, 763 c.c. sea-water placed in Flask No. 4 acting as a control. Under the conditions of the experiments with air bubbling through both flasks and thermocouple junctions in position, and the flasks sunk in the water of the thermostat, k for the first flask was 0·0238, and for the second $k = 0{\cdot}0236$. Temperature adjusted all round to within a hundredth of a degree to 14·5° C. Cooled sperm added and readings started.

Time	3.30 P.M.	galvanometer scale	reading	0	
„	6.30 „	„	„	17 mm.	R flask containing eggs.
„	9.30 „	„	„	26	„ „ „ „
„	12.30 A.M.	„	„	47	„ „ „ „
„	2.30 „	„	„	59	„ „ „ „

At end of experiment 95–100 per cent. of eggs were in healthy free-swimming early gastrula stages. Kjeldahl determination gave 146·2 mgrm. of egg nitrogen present in flask contents. The heat liberated in first hour 6·34 grm.-calories, in the fifth hour 28 grm.-calories, and in the eleventh hour 74·4 grm.-calories (see Curve, fig. 2, *a*).

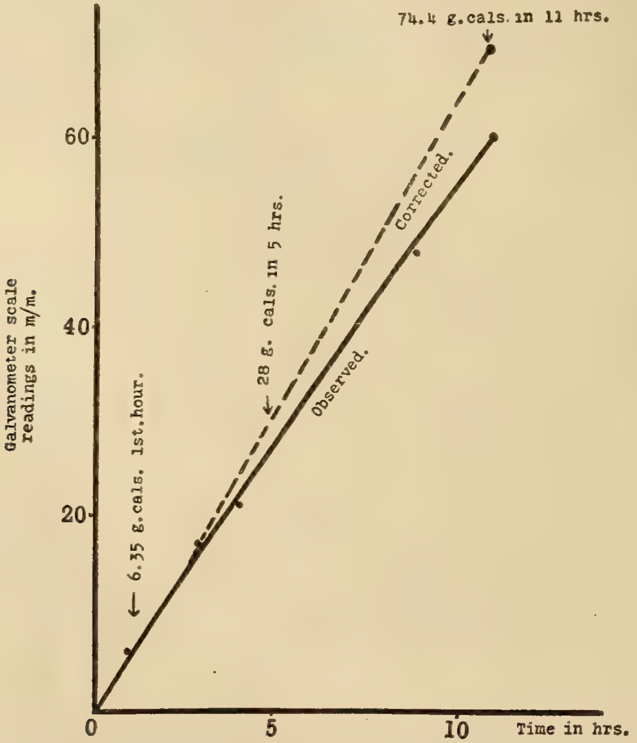

FIG. 2, *a*.—Curve of heat production to Experiment II. 146·2 mgrm. egg nitrogen.

(b) *Oxygen and CO_2 Determination.*—2 c.c. of same lot of egg material as that put in the flask in the first part of the experiment, placed in a chamber

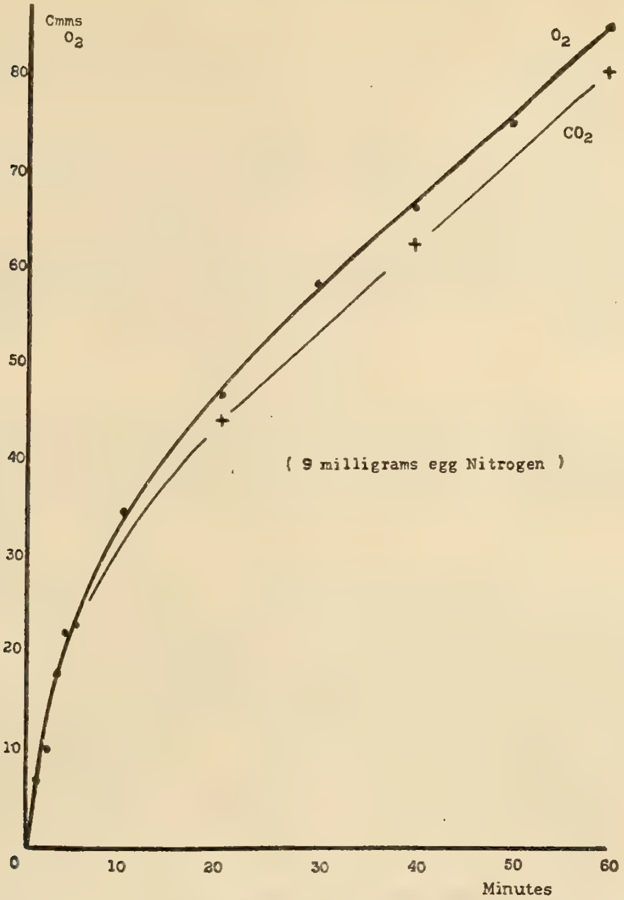

FIG. 2, *b*.—Curve of O_2 consumption and CO_2 liberation for Experiment II. 9 mgrm. egg nitrogen.

of oxygen manometer, and the same quantity in a chamber of CO_2 apparatus. The eggs were fertilised. Those in the oxygen manometer showed that at the end of

1	minute	after addition	of the sperm	eggs had	consumed	6·95	c.mm.	oxygen.
2	minutes	"	"	"	"	10·0	"	"
3	"	"	"	"	"	18·0	"	"
4	"	"	"	"	"	22·0	"	"
5	"	"	"	"	"	23·05	"	"
10	"	"	"	"	"	34·5	"	"
20	"	"	"	"	"	47·0	"	"
30	"	"	"	"	"	58·3	"	"
40	"	"	"	"	"	66·2	"	"
60	"	"	"	"	"	85·0	"	"

At end of experiment 100 per cent. of eggs showed fertilisation membranes and commencing two-cell stage. Kjeldahl determination gave 9 mgrm. egg nitrogen present.

At this rate 146·2 mgrm. egg nitrogen would consume 1380 c.mm. oxygen in the first hour following fertilisation, 1380 c.mm. oxygen being equivalent to 1·97 mgrm.

The heat produced by 280 mgrm. egg nitrogen in the first hour following fertilisation,, as shown in the early part of this experiment, was 6·35 grm.-calories. So that

$$Q = \frac{6{\cdot}35}{1{\cdot}97} = 3{\cdot}22.$$

The heat production rose in the fifth hour to 28 grm.-calories, and to 74·4 grm.-calories in the 11 hours in this experiment. The corresponding CO_2 determination for this experiment gave a respiratory quotient of 0·95.

Experiment 3.

800 c.c. of ripe well washed *E. miliaris* eggs were placed in Flask No. 3, 763 c.c. plain sea-water being placed in Flask No. 4, acting as a control under the conditions of the experiment with flasks sunk in the water of the thermostat and air-bubbled through both flasks, k for flask No. 3 was 0·0238 while that for Flask No. 4 was 0·0236. Temperature was adjusted all round to within a hundredth of a degree to 14·5° C. No sperm were added. Galvanometer readings were commenced. At the end of 1 hour Flask No. 3 had given off 3·6 grm.-calories of heat. A Kjeldahl determination on the flask contents gave 431 mgrm. egg nitrogen present. In the same time 8 mgrm. egg nitrogen of the same batch of egg material, consumed 15·1 c.mm. oxygen. Therefore 431 mgrm. of egg nitrogen would consume at this rate 812 c.mm. oxygen in this time, as 812 c.mm. oxygen equal 1·17 mgrm. O_2 we get value of

$$Q = \frac{3{\cdot}6}{1{\cdot}17} = 3{\cdot}07$$

for the unfertilised egg. The value obtained for the fertilised egg of *E. miliaris* in the two previous experiments was 3·215 and 3·22 respectively. Thus the value of Q is somewhat different in the two cases.

It is doubtful, however, if much significance can be attached to this difference. The gonads of so many females have to be used for making determination on the heat production of the unfertilised egg, that it is impossible that they should all be in the same stage of ripeness. Some of the gonads are certain to be slightly over ripe, and their eggs will probably cytolyse on being placed in the vacuum flask, and will give off an abnormally large amount of heat; others, again, will be somewhat immature, and will consequently give off little heat as compared with the properly mature stage. On the whole, from a number of experiments with the unfertilised egg, I am inclined to think there is little difference between the value of Q in the unfertilised, as compared with the fertilised egg, and that Meyerhof's conclusion that it is the same for both is correct. In three successful experiments with the unfertilised egg, the figure for Q obtained in the above described experiment represents the mean, and it is worth noting that this value is slightly smaller than in the case of the fertilised egg.

Discussion.

The Unfertilised Egg.—In the foregoing experiments it has been shown that the oxygen consumption and the heat liberation of the unfertilised egg of *E. miliaris* is remarkably small. In 1 hour, 1,000,000 eggs (8 mgrm. egg nitrogen) only consumed 15·1 c.mm. of oxygen, and liberated at the same time something of the order of 0·067 grm.-calorie heat. If we divide the heat liberation in 1 hour's time, expressed in gramme calories, by the oxygen consumption in milligrams, we get a quotient which we may call the calorific quotient. In the case of the unfertilised egg this quotient was found to be 3·07. Meyerhof, in *Strongylocentrotus*, using the Winkler method for estimating the oxygen consumption and the direct method for measuring the heat production, found this egg consumed 9·41 c.mm. of oxygen and liberated 0·038 grm.-calorie, under similar conditions. The value of Meyerhof's calorific quotient (Q) varied about 2·8.

The Fertilised Egg.—On the addition of the sperm to the eggs of *E. miliaris*, there is an immediate oxygen consumption by the egg and a corresponding increase in the heat liberated. At the end of the first hour of development 86·4 c.mm. of oxygen were consumed by 1,000,000 eggs (8 mgrm. egg N.), and 0·397 grm.-calorie of heat was liberated. The CO_2 output of eggs was almost the same as the oxygen intake; the respiratory quotient being in the vicinity of 0·92. In *Strongylocentrotus*, Meyerhof found under similar conditions for 65·38 c.mm. of oxygen consumed a heat liberation of 0·247 grm.-calorie, for a similar quantity of eggs. The calorific quotient in this instance again being 2·6 to 2·8. In view of the large number of eggs that have to be used for making a heat determination on the unfertilised egg, it is doubtful if much significance attaches to the difference found between the calorific quotient of 3·07 in the unfertilised egg, as compared with 3·2 in the fertilised condition.

Summary.

1. In the present paper an attempt has been made to measure the oxygen consumption of the egg of *Echinus miliaris* on fertilisation and early development and compare it with the amount of heat liberated by the egg at the same time.

2. In making both these estimations new methods have been employed.

3. The oxygen consumption of the egg has been measured by the use of a special pattern of the Barcroft differential manometer, in which the eggs were fertilised within the closed chambers of the apparatus. The CO_2 output of the egg was also measured with the same instrument and the respiratory quotient determined.

4. The heat liberation was measured by the use of the differential micro-calorimetric method.

5. In 1 hour 1 million unfertilised eggs (8 mgrm. egg N.) consumed 15·1 c.mm. of oxygen and gave off at the same time 0·067 of a gram-calorie of heat at standard pressure (760 mm. Hg.) and temperature 14·5° C.

6. In the same time the same quantity of fertilised eggs consumed 86·4 c.mm. oxygen (with a corresponding output of CO_2, respiratory quotient 0·92) and liberated 0·3976 of a gram-calorie of heat under similar conditions.

7. The fertilised egg in the first hour of development gave off roughly 6—7 times more heat than the unfertilised egg and consumed at the same time 6 or 7 times more oxygen than the unfertilised egg.

8. In the fertilised egg in one experiment (I) 58·4 mgrm. egg nitrogen (about 7·3 million eggs) liberated 2·9 gram-calories at the end of the first hour of development; in the fifth hour, 10·5 gram-calories and 22·8 gram-calories of heat in 11 hours. In another experiment (II) 146·2 mgrm. egg nitrogen (18·6 million eggs) liberated 6·35 gram-calories in the first hour, 28 gram-calories in the fifth hour, and in 11 hours 74·4 gram-calories. (This last figure is possibly too high, due to some cytolysis.) On the whole the heat liberation of the egg on fertilisation rises steadily, reaching its highest point when segmentation has been completed and the free-swimming stage is reached.

9. The heat liberation of the egg during the first hour after the sperm have been added to the eggs expressed in gram-calories divided by the amount of oxygen consumed in the same time, expressed in milligrammes, gives a calorific quotient (Q).

10. In the case of the unfertilised egg the calorific quotient was found to be about 3·07, while in the fertilised egg it was found to be 3·22.

11. On fertilisation a greatly increased liberation of chemical energy is brought about within the ovum. This is shown by the increased oxygen consumption of the fertilised egg-cell combined with its greatly increased carbon dioxide and heat liberation.

12. As, however, the calorific quotient of the unfertilised and the fertilised egg-cell is approximately the same in both instances, little or almost a negligible quantity of this energy is expended in bringing about the visible morphological structure of the developing ovum. It is probably employed in keeping the living substance itself intact as a physical system.

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Further Observations on Cell-wall Structure as seen in Cotton Hairs.

By W. Lawrence Balls, M.A., Sc.D. (Cantab.), and H. A. Hancock.

(Communicated by Dr. F. F. Blackman, F.R.S. Received March 3, 1922.)

[Plate 10.]

The present note summarises the results of observations made subsequently to the recognition of growth rings in the cell-wall,* of which a photograph in transverse section is given in Plate 10, fig. 2. These observations are all related to previous physiological studies, and most of them have been made on material of known origin, *i.e.*, dated bolls† and pure-line plot crops.

An excellent memoir on our present knowledge of the cotton cell-wall by Mr. H. J. Denham,‡ now in course of publication, makes it unnecessary for us to deal with the historical aspects of the matter.

Methods.

(*a*) The "swelling" technique has been further developed by the use of calcium thiocyanate (for which, as well as for the use of naphthamine blue as a stain, we are indebted to Mr. H. E. Williams§). Other reagents have also been found serviceable when the conditions are adjusted to produce an equilibrium state on the verge of actual solution, *e.g.*, cuprammonium and caustic soda, alone or together, and also sulphuric acid have been largely used. The latter is exceptionally interesting as showing specific differences between various hairs in respect of the critical concentration. The artifact nature of swollen walls has been continuously borne in mind, and all observations have been returned to the unswollen state by measurements of the contractions and expansions experienced by the hair.

(*b*) Our section-cutting technique has been described elsewhere.||

(*c*) The junior author devised a simple "pressure" technique, single hairs

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* W. L. B., "Existence of Daily Growth Rings in the Cell-wall of Cotton Hairs," 'Roy. Soc. Proc.,' B, vol. 90 (1919).

† W. L. B., "Raw cotton (Development and Properties of)," chapter 4 (London, 1915).

‡ Denham, H. J., "The Structure of the Cotton Hair and its Botanical Aspects: Memoir of the British Cotton Industry Research Association," 'Jour. Textile Inst.,' vol. 13, p. 99 (1922).

§ Williams, H. E., "The Action of Thiocyanates on Cellulose," 'Jour. Soc. Chemical Industry,' vol. 40, p. 221 T (1921).

|| Denham, H. J., 'Nature,' vol. 107, p. 299 (1921); W. L. B. and H. A. H., 'Nature,' vol. 107, p. 361 (1921).

being stressed enormously, under a cover-slip, by pressing with the blade of a knife. This we have found very useful; it is evidently akin to the breaking of aeroplane timber studied by Robinson.*

(*d*) The inter-relation of external convolutions and internal wall structures has been systematically examined by detailed repeated mapping along the length of single hairs.

(*e*) Groups of hairs of equal length from single seeds have been similarly mapped to obtain the average distribution of convolutions in studying the change from day to day in dated samples. A very full re-examination of the daily pickings samples† has also been made in connection with this work but only slight use will be made of these results at present, as they need direct experiment on growing plants for confirmation of our interpretations.

The evidence on which we base our conclusions is very detailed and various, and to particularise every item would be extremely tedious. We have, therefore, adopted the plan of summarising our results rather after the manner of geological research; and by way of further assistance in keeping this note from undue expansion, we shall restrict ourselves to dealing with the more debateable features of hair structure as brought out by Mr. Denham's memoir, already referred to.

The Cuticle.

This we found to be distinct from the primary wall, though extremely tenuous. It possesses a spiral structure, probably showing reversals of the spirals, and quite probably identical in frequency, or even in details of pattern, with the pit spirals (see below), but the difficulty of correlating the two sets of observations is very great; Haller's method‡ ($SnCl_2$ and $AuCl_3$) was not successful. The spiral lines of weakness and their apparent reversals, plus the resistance of the cuticle to solvents, determine the familiar "beading" of swollen hairs. The miscalled "stomata" of De Mosenthal§ are probably primary wall-structures in essence, the cuticle being moulded to them; we doubt very much whether the cuticle is actually perforated. A granular superficial structure seen after heating (in the thiocyanate process) and staining with osmic acid seems to be due to the melting and redistribution of the wax film, which varies in amount with varieties and species of cotton around 0·4 per cent. of the hair weight.

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* Robinson, W., "The Microscopical Features of Mechanical Strains in Timber and the Bearing of these on the Structure of the Cell-wall in Plants," 'Phil. Trans.,' B, vol. 210, p. 49 (1920).

† W. L. B., "Raw Cotton," p. 112, *loc. cit.*

‡ Haller, 'Chem. Zentr.,' p. 652 (1920).

§ De Mosenthal, H., "Observations on Cotton and Nitrated Cotton," 'Jour. Soc. Chem. Ind.,' vol. 23, p. 292 (1904).

The Primary Wall.

Various details of our evidence confirm the view that this wall, even when adult, is a different cellulose from that of the secondary wall. Further, we have seen reason to believe that until growth in length has passed its maximum rate, the cellulose (as distinct from the cuticle) has a different composition from that which it has assumed when the secondary thickening begins. We ourselves, for convenience, called this early stage "pre-cellulose," and we have since learned* that the general problem is now being investigated by Priestley. Accidentally, our preparations have shown the secondary cellulose completely dissolved, but the primary wall, spirally marked, untouched.

The pit spirals of the secondary wall (see below and figs. 5–7) are continuous through the primary wall, and possibly even to the cuticle spirals (*vide supra*). Thus it would seem that the law of Predetermination† plays an important part in the cotton hair, and the bearing of this on our convolution maps will be described later.

The objects discovered and excellently photographed by De Mosenthal‡ we propose to designate as the "slow spirals." Their nature is obscure, but they are evidently a kind of pitted corrugation in the outer surface of the primary wall, to which the cuticle moulds itself. The sides of the trough in which the elliptical craters lie often project beyond the surface of the hair, and are thus discernible in profile. These slow spirals are particularly easy to see in fuzz hairs, but are probably present to some degree along all parts of every hair, lint (fig. 3), or fuzz, for they flash out momentarily during swelling with critical-strength sulphuric acid, even though not visible previously. The spiral shows frequent reversals (fig. 3). The relation of this spiral to the pit spirals, in respect of pitch, direction, and reversals, has been studied in detail, and we have satisfied ourselves—with some regret for an untenable hypothesis—that they are sometimes independent of one another. We have no evidence that the slow spiral pattern is pursued into the formation of the secondary wall, unlike the pit spirals, nor even that it represents any textural modification (as distinct from modification of surface or thickness) in the primary wall itself, excepting that when a hair has been "tendered" by acid it shows a saw-tooth form of cracking, the long slope coinciding with pit spirals and the short resembling slow spirals (fig. 4). However, as we have satisfied ourselves that these spirals are not invariably opposed in direction, the fact may not be relevant, though it does suggest a chemical polarity.

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* Discussion in Section K on the "Quantitative Analysis of Plant Growth," 'British Association,' 1921.

† W. L. B., "Predetermination of Fluctuation, a Preliminary Note," 'Proc. Cambridge Phil. Soc.,' May, 1914.

‡ *Loc. cit.* W. L. B., "Analyses of Agricultural Yield," Part III, 'Phil. Trans.,' B, vol. 208, p. 157 (1917–18).

These slow spirals are thus somewhat mysterious objects, and we must wait for direct growth experiments to elucidate their nature. In passing, we may note that the distortions produced by swelling make the quick pit spirals simulate them very closely (fig. 8), a circumstance which led us to much confusion at first.

The Secondary Wall.

One of us has formerly described the growth rings,* while many observers have figured and commented on the existence of spiral markings in the wall and on its inner surface especially. The occurrence of somewhat elusive simple pits has also been described from fresh material by the senior author,† and the probable existence of an internal spiral structure has been suggested in various forms by non-botanical writers.‡ We are now in a position to co-ordinate all these observations and views.

By means of the simple "pressure" method, it is possible to "develop" a spiral series of cracks throughout the length of a hair, with little distortion, which can then be mapped in detail (fig. 6), and related to a previous charting of the external form of the hair (*vide infra*). Such a pressed hair dissolves much more quickly in swelling reagents, presumably from the greatly increased free surface, and possibly also (since lower concentrations will attack it), because the flank of the patterned and orientated cellulose aggregates is, so to speak, exposed. When swollen, only a complex structure resembling basket work is produced (fig. 5), and it was the spasmodic occurrence of this in slides given us by Mr. Williams§ which started the present research.

By mapping the simple pits in fresh greenhouse material, for which we are indebted to Mr. Vernon Bellhouse, and then mapping their spiral cracks, we have satisfied ourselves that the pits are simply abnormally wide intervals between otherwise contiguous spirals. It is therefore possible that any plant cell-wall which shows simple pitting may possess spiral structures similar to that of the cotton hair. We would also call attention to the structures in wood cell-walls described by Robinson,‖ who describes and figures these so-called slip planes as being interrupted by the fragile middle lamella, which seems improbable unless these "slip-planes" are pre-existent. The spasmodic occurrence of swollen spirals in thiocyanate preparations merely showed an erratic anastomosing series, but with the cuprammonium and soda mixture, and better still with critical sulphuric acid (circa 1·540 sp. gr.), the nature of these anastomoses was evident; the wall is then seen to consist of about a hundred spiral fibrils—a screw of a hundred threads—all approximately identical, except in one respect. This exception consists in the frequent presence of two spirals, within the series, which stain more deeply than the others with naphthamine blue. They do not seem to be otherwise different from their neighbours in any way, and as they seem always to lie at the ends of the major axis of the collapsed cell-wall, they might be merely stress-produced artifacts. On the other hand, one of them may disappear by approaching the other, the interval between them changing. Thus, the appearance shown in swollen hairs is altered from a symmetrical double screw (figs. 8, 9) to an asymmetric one, and thence to a single-thread screw, as we pass along the hair. This is not compatible with artifact origin, and it would seem that for some reason unknown two fibrils, lying diametrically opposite one another, are somewhat different from the others. We have noticed similar bifurcation and re-union in the spiral thickening of protoxylem vessels in other plants.

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* W. L. B., "The Existence of Daily Growth Rings in the Cell-wall of Cotton Hairs," 'Roy. Soc. Proc.,' B, vol. 90.

† W. L. B., "Raw Cotton," fig. 12 and p. 78.

‡ *Vide* Denham, Memoir, B.C.I.R.A., *loc. cit.*

§ Williams, *vide supra.*

‖ Robinson, *loc. cit.*

The question of the relationship between these radial boundary surfaces and the tangential growth-ring boundaries next arises. Numerous attempts to demonstrate the matter clearly in transverse section have largely failed; and some considerations relating to free surface, cohesion, and the like, make it rather unlikely that these two sets of structures could ever be thus demonstrated perfectly and simultaneously in the swollen state. We also suspect that the shearing stress of the razor edge may produce molecular disturbances which alter the reactions of the cellulose. Partial demonstrations of the existence of these radial boundaries have frequently been obtained, but rarely (fig. 1) comparable with the growth-ring demonstration of fig. 2. We thus have to depend on optical longitudinal sections, and by this means have satisfied ourselves in exceptionally good preparations that the spirals are arranged in layers, each layer constituting a single growth-ring.

The cotton hair cell-wall is thus an elaborate structure, laid out on a simple plan. In the first instance, a spiral pattern seems to be laid out in the primary cell-wall and cuticle; this, it should be noted, must happen while the hair is growing in length. The deposits of secondary cellulose, as growth-rings, do not obliterate this pattern, but follow it most strictly. Thus, a radial (spiral) structure persists through the concentric deposits. The simple pits of any cell, equally with those of cotton hairs, are, after all, merely a special case of the same procedure, while an analogy may be found in the medullary rays of timber.

Our partial elucidation of this structure has already thrown light on some physical properties of the hair. Abnormal hairs are often found in which, to a greater or less extent, the spiral structure is visible without any "development." Our colleague, Mr. Slater, in the Physical Section of this Department, has, in some preliminary studies, found the flexibility of such hairs to be highly abnormal, such hairs standing in the same relation to normal hairs, as strands of yarn compared with solid wires of celluloid. We have mentioned our opinion that the razor edge may produce molecular disturbances in the cellulose. Akin to this is a remarkable phenomenon discovered by the junior author, reminiscent of the results described by Griffith* with quartz rods. If a hair has been pressure-treated to develop the pit spirals without re-agents and then is subjected to stress in longitudinal extension, no alteration is noticeable until the hair breaks; after breaking, however, little or no trace of the pit spirals is left in any part of the hair. We have failed to obliterate the spiral cracks by any tension without actual breakage, and it would seem that, as in Griffith's work,† a molecular disturbance is needed, which the back-lash of the break provides.

Dimensions and Constitution of a Pit Spiral Fibril.

Taking $0 \cdot 4\mu$ as the thickness of a substantial growth-ring, and allowing 100 spirals to the ring in a hair whose original cell diameter was 15μ, and its mean wall diameter considerably less, gives us $0 \cdot 4\mu$ square as the approximate cross-sectional area of one Pit Spiral Fibril. Its length is apparently that of the hair. Without undue speculation it is evident that we are here approaching molecular dimensions, the probable size of the cellulose molecules being such that some number of them between 1,000 and 100 would constitute the cross-sectional area of one such pit spiral. There is even the slight possibility that in these pit spiral fibrils we have reached the limits of morphology and are examining a chemical (or colloido-chemical) unit. For other reasons, however, we rather incline to the view that "cellulose," even in a pit spiral fibril, is a complex of more than one kind of cellulose molecule.

Origin of the Pit Spiral Structure.

It seems clear to us that this secondary wall structure is a predetermined one, and that, paradoxically, we must therefore look to the period of growth in length to determine the causation of such abnormalities in it as we have mentioned. Physiological work in this direction, under glass, is contemplated. But this leaves unsolved the more fundamental problem, as to why even the primary wall should be thus patterned and heterogeneous, consisting of structures which can sometimes be mechanically broken apart, which are possibly only united by a molecular film of water, and which show (when enormously swollen and stained with naphthamine blue) granular lines alternating with clear zones (fig. 8).

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* Griffith, A. A., "The Phenomena of Rupture and Flow in Solids," 'Phil. Trans.,' A, vol. 221, p. 163 (1920).

† Griffith, *loc. cit.*

Until a late stage we were not certain whether the slow spirals were not always opposed in direction of rotation to the pit spirals, and a working hypothesis was adopted which combined Church's results* on the fundamental geometric structure of the cell with a general idea of protoplasmic circulation, and with some earlier studies of growth in a fungus hypha by one of us.† This hypothesis, though now entirely speculative, may yet be of interest. It postulated the existence of two "growth centres" mutually exclusive or polar in their inter-relations, as a rule; these controlled the longitudinal extension of the cell by intussusception, and their micro-bio-chemical operations were rhythmic, as in Liesegang ring-formation. It was unlikely that such a system would build forward along a straight line, hence revolution was postulated, sometimes right-handed, sometimes left-handed (fig. 3), under the influence of accident or environment or even of stereo-isomerism. This revolution of the builders produced the spiral form, their rhythmic operation the successive fibril phases; and molecular predetermination, akin to crystallisation, produced fibrillar continuity.

When it became clear that the slow spirals were not the trails of these "growth centres"—since their direction was not invariably opposed to that of the pit spirals—the hypothesis became mere speculation.

It does, however, remain clear that there is a fundamental geometric structure in the cell wall, though our hope of confirming and extending Church's main conclusion has at present failed.

The Protoplasmic Débris.

We have only to mention that this is of assistance in swelling technique as a rough guide to the amount of longitudinal contraction.

The Convolutions.

We now come to a much observed feature of the cotton hair, about which nothing definite has been published, whether in respect of their effect on spinning properties, or on the physical properties of the hair, or of their origin. The senior author has regarded them* as a necessary consequence of the simple pits in the wall, with modifications caused by wall-thickness variations, and we can now extend this to include the pit spiral structure, which should completely explain the convoluted form of the collapsed dead hair.

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* Church, A. H., "Phyllotaxis in Relation to Mechanical Laws" (Oxford University Press, 1904).

† W. L. B., "Temperature and Growth," 'Ann. Bot.,' 1909.

Actually, however, the explanation is not yet complete. Mapping pit spiral against convolutions, in any one piece of hair (fig. 10), there is a general similarity, but by no means exact identity. It is probable, however, that if we could construct scale models of the wall structure (growth rings and pit spiral fibrils) in the form of semi-rigid tubes, and then cause them to collapse, we should find that local variations of texture, packing, interfibrillar friction, wall-thickness and hardness, etc., would produce similar discrepancies to those we have observed, and we venture to think that the existence of convolutions can be explained in this way, if we include the reinforced spirals already mentioned, whose presence makes the "arch" structure unsymmetrical.

This, however, leaves a major problem to solve, to wit, the reason why the pit spirals, and hence the convolutions, vary their pitch and direction. On grounds of convenience, we have studied the convolutions rather than their causative spirals, and while no definite conclusions have yet been reached, a number of suggestive observations have been made on the daily pickings material,† which consists of fruit-capsules (bolls) opening on ninety successive days:—

(*a*) On counting the number of convolutions in a unit length at the middle of the hair (fig. 11, heavy line), we found the average number changing from day to day, as in the case of other hair properties, indicating that environmental changes affected the convolutions. Similar results were given by counting at other *loci* (fig. 11), and by taking the percentage distribution as between these various *loci* (fig. 12).

(*b*) Extending these measurements by mapping the convolutions along the whole length of the average hair (fig. 13), there were indications that the *locus* of any feature (*e.g.*, a low number of convolutions) shifted its position along the hair on successive days. The earlier the day of boll opening the further removed was the particular feature from the base of the hair, thus indicating that the environmental determinant of convolution form must have operated while the hair was still growing in length. Our evidence is based on too slight data to be conclusive in itself, but it will be noticed that it confirms

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* W. L. B., "Raw Cotton," pp. 79 and 147, *loc. cit.*

† *Vide supra.*

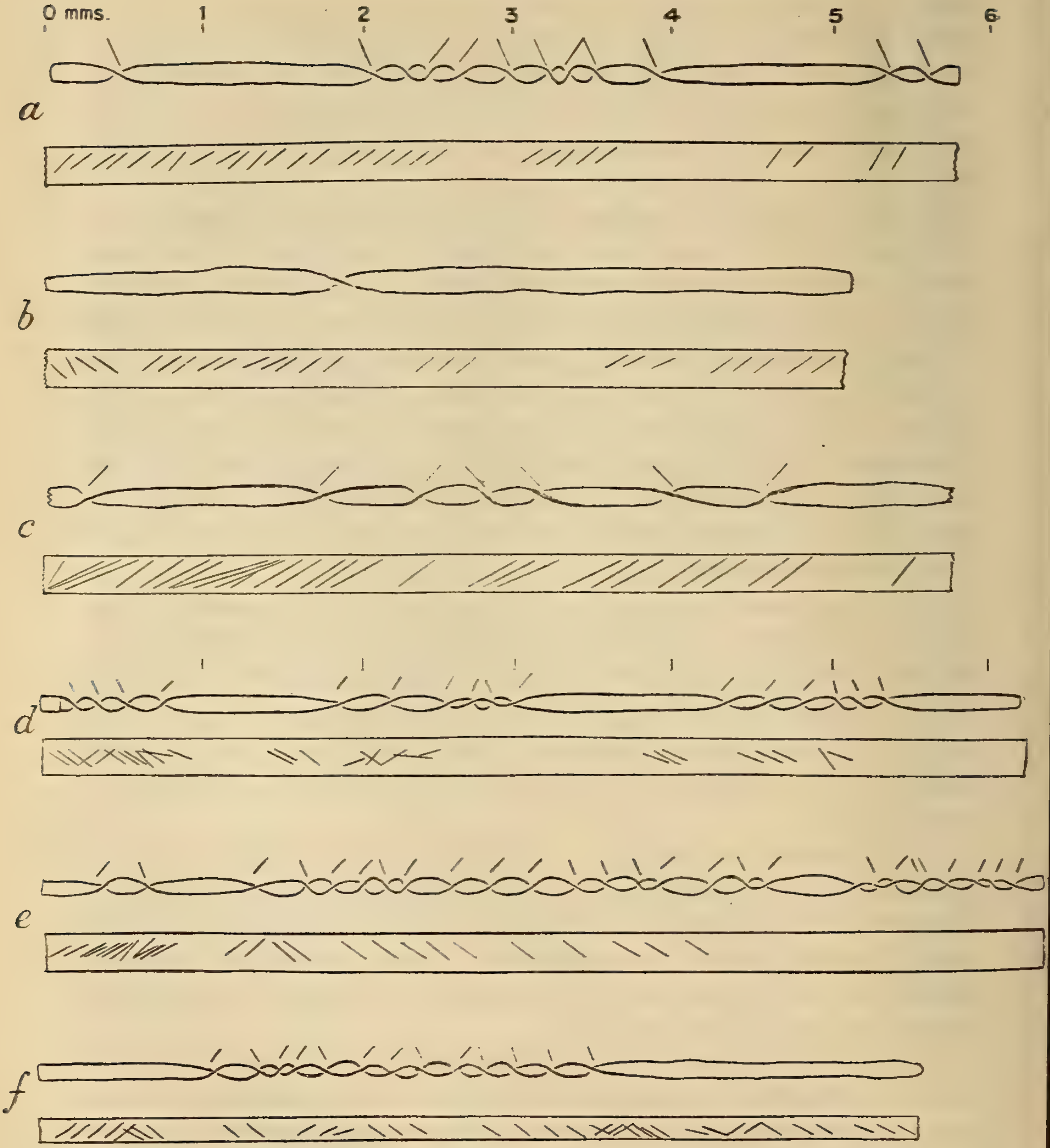

FIG. 10—*a* to *f*.—Each pair of drawings represents one piece of hair and shows:—above—the position of the convolutions with slanting lines drawn to indicate the direction at each turn; below—direction of slope of pit spirals, as determined at various points after pressing. Length corrected to original value. Exceptionally discrepant pieces have been selected for reproduction.

our conclusions already drawn from microscopic work, and thus increases their probability.

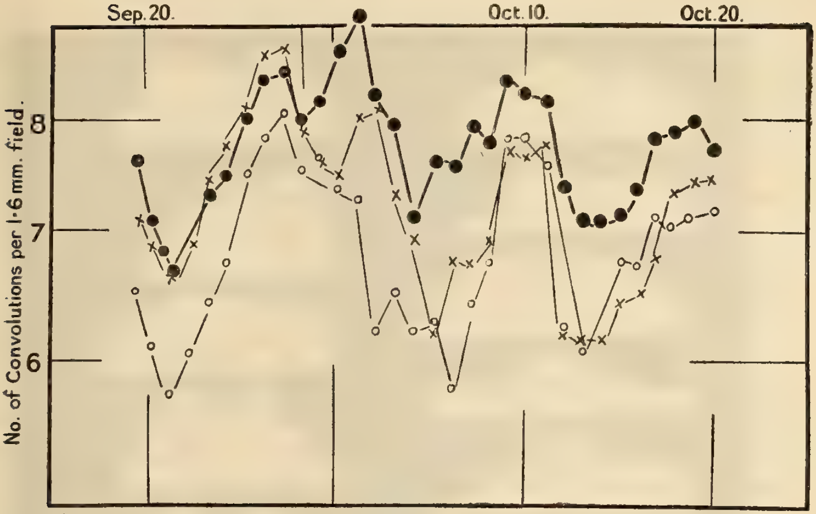

FIG 11.

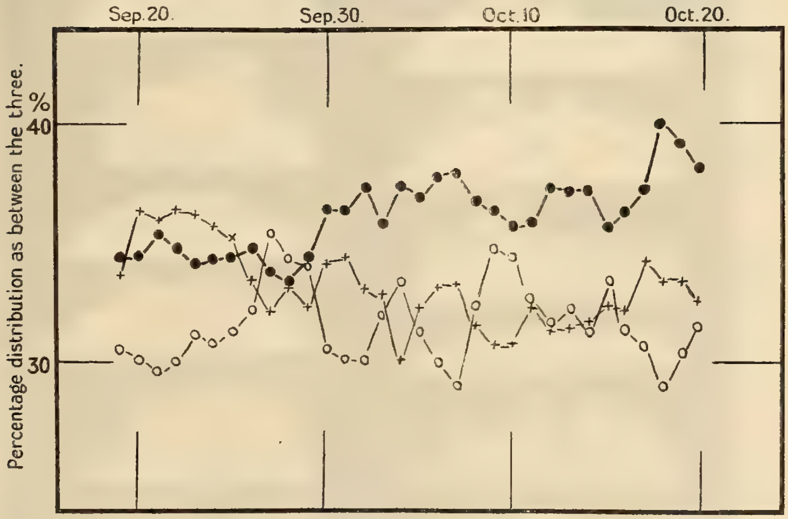

FIG. 12.

FIGS. 11 and 12.—From capsules opening on successive days in the daily pickings series (q.v.) five seeds were taken each day at random, and twenty hairs of similar length from each of these five were arranged with their bases in line. The number of convolutions included in a microscope field of 1·6 mm. was then determined at three *loci* along the hair, respectively, 5, 15 and 25 mm. from the base. The curves as shown are the three-point means of the original data, each point thus representing the average of 300 hairs. The data are further analysed in fig. 12 in order to discriminate between general fluctuation common to the whole length of the hair, as indicated in fig. 11, and the existence of differential fluctuation in various parts, due to determinations taking place at different times during development. The logarithmic plotting shows that the latter fluctuation is quite marked, though its amplitude is reduced by a half.

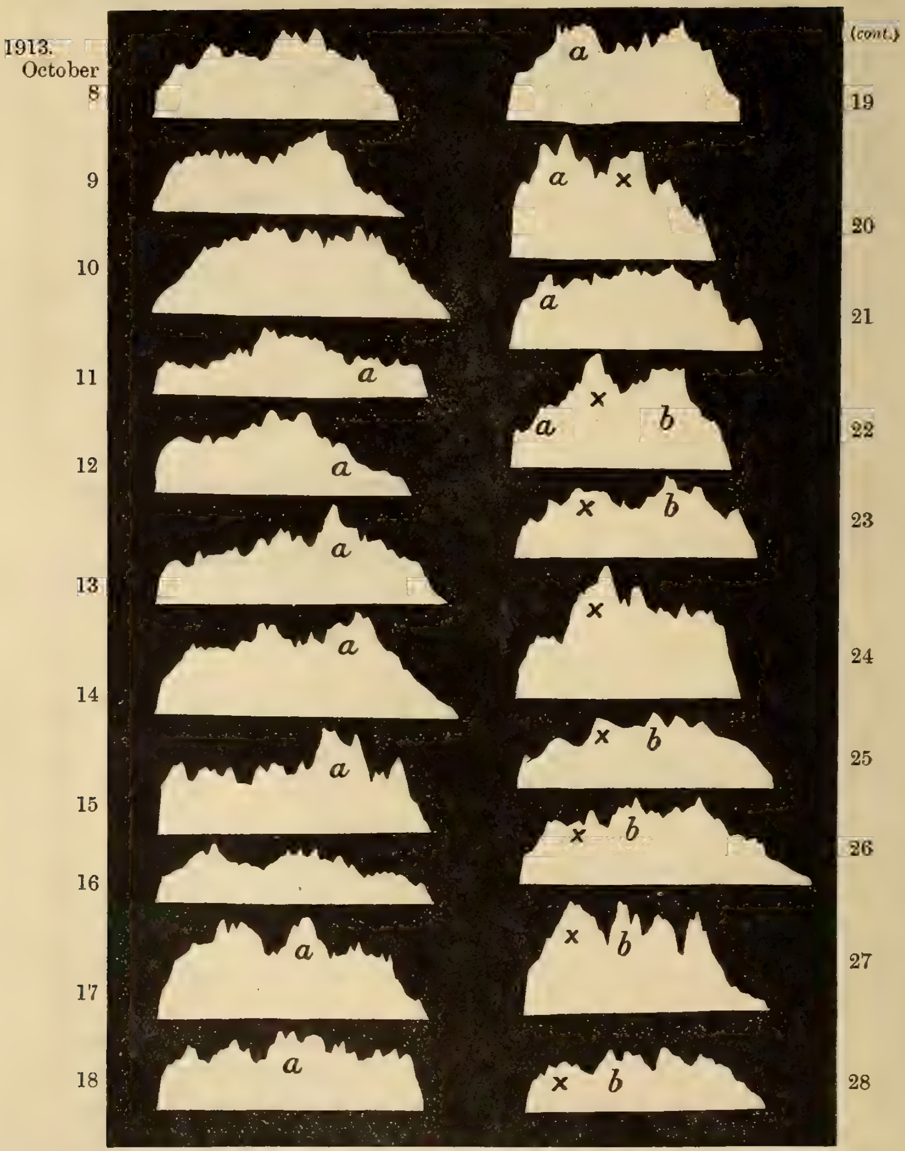

FIG. 13.—Average distribution of convolutions from day to day along the whole length of the hair. Data obtained by taking twenty hairs from the same square millimetre of one seed chosen at random each day. These hairs, being roughly of the same length, were arranged with their bases in line and the number of convolutions in a 1·6 mm. field measured at intervals of successive half millimetres. In five cases, where only twelve hairs were measured, the scale of the distribution curve has been corrected accordingly. The letters *a*, *x* and *b* are placed on some of the curves to indicate the general tendency which appears to be shown, though indistinctly, by the various modes, in shifting backwards towards the base as the date of opening of the capsule becomes later. Base of hair at left hand of each curve.

The actual data were obtained by selecting usually twenty hairs of equal length on each day over a sequence of thirty days, and counting the convolutions in successive intervals of 1·5 mm. from base to tip; they thus comprise some 20,000 measurements, but statistical considerations make it evident that they need to be greatly extended in order to be conclusive, and in practice it should be found easier finally to attempt the proof by means of direct physiological experiment and by observations of the pit spirals.

A fairly close correspondence was indicated as between the general form of the hair-length growth curve (formerly ascertained)* and the shift of any convolution-form *locus* from day to day.

(*c*) A renewal of a former attempt was also made, in order to see whether any forms or markings could be found along the length of the hair which would correspond for linear extension to the daily marks of the growth-rings in secondary thickening. The system of convolution mapping was extended to measure the length and direction of every separate convolution. These measurements were then plotted as shown (fig. 14), using rectangles of equal area for each one, the bases of which were equal to the convolution length, and in this form of plotting it is very evident that the convolution sequence along any one hair is at least wave-like. Phases of steep-pitched and short convolutions alternate with phases of slow and long convolutions. The discrepancies between convolutions and pit spirals seem to happen chiefly in the latter phase, which also seems, as might be expected, to contain more convolution reversals (fig. 14, *b*). The number of peaks (short convolution phases), in the curve, seems to tend towards correspondence with the number of days (about 25), during which the hairs used in these observations (daily pickings samples) were growing in length.

Here, again, no rigid conclusion can be drawn, but the facts are certainly very suggestive of a daily environmental effect, acting by predetermination on the convolutions through the pit spiral patterning of the primary wall.

(*d*) We have not forgotten the probability that mutual pressure inside the growing capsule, together with the curling grouping which the hairs thereby take up, may influence both pit spiral and convolution, but we anticipate that this will be found subsidiary to the other causes indicated.

(*e*) A source of error in other observations made on material which had been acted upon by softening and swelling re-agents should be noted. When the wall cellulose is softened with the hair held in slight tension, the convolution spiral may become mechanically unstable and instantaneously jump to a new conformation, the hair becoming a cylindrical helix, like an

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* W. L. B., "Raw Cotton," p. 76.

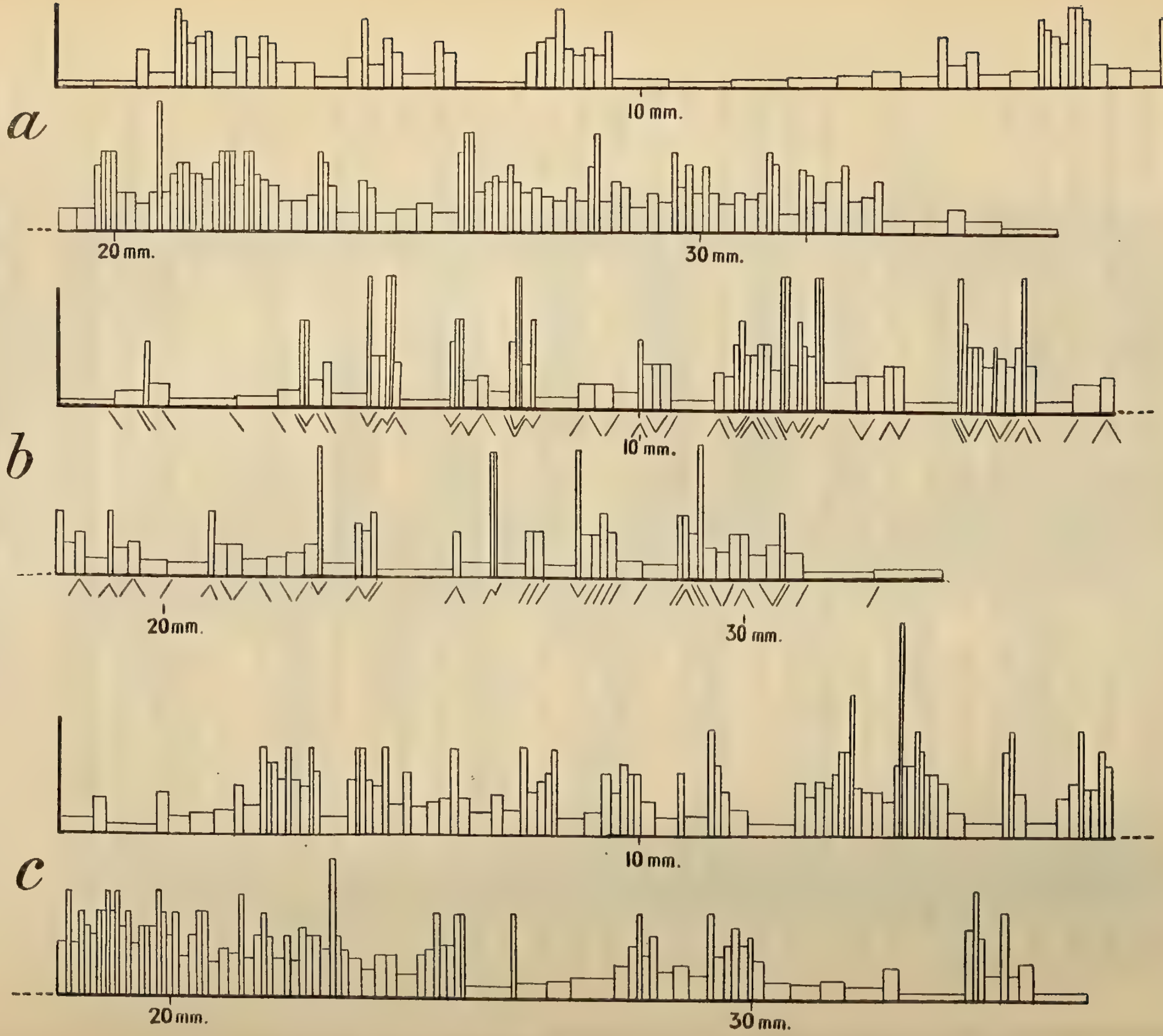

Fig. 14 shows the position of every convolution along three hairs, *a*, *b*, *c*. As also in fig. 13, the observations were made in air. The form of the linear distribution is made more evident by plotting rectangles of equal area over every convolution. In the case of fig. 14, *b*, a series of sloping lines drawn below the base line indicates the direction of each convolution in addition to showing its length as in 14, *a*, and 14, *c*.

"Ayrton spring." This change is apt to complicate observations on wall structure.

The Fuzz Hairs.

In a previous communication,* one of us has pointed out that fuzz and lint appear to be identical in all respects, except that growth in length is inhibited in fuzz, while growth in wall thickness is restricted in the lint. None of the present observations have revealed any further differences. In many cases we have found it convenient to try out new methods, or hypotheses, on the fuzz hairs before attempting to apply them to the more delicate lint.

Conclusions.

1. A spiral fibrillar radial structure exists in every growth-ring of the cell-wall of the cotton hair.

2. The simple pits of the cell-wall are a special case of this general structure.

3. The pattern of the spiral appears to be predetermined during growth in length.

4. This pattern is preserved through all the growth-rings of the secondary wall thickening.

5. The number of fibrils in cross section of one hair is of the order of 1,000 upwards.

6. The pattern (direction, reversal, and pitch) of these spirals seems to be the major determinant of the externally visible convolutions of the hair.

7. There are indications that the unknown cellulose-aggregates, which compose any one spiral fibril, have a definite geometric conformation, suggestive of stereo-isomerism.

8. Attempts to elucidate the cellulose-structure further, as by X-rays, will probably have to take account of this spiral fibril arrangement.

While assistance in various ways has been given by our colleagues in the Experimental Department of the Fine Cotton Spinners' and Doublers' Association, to whose Executive Directors we are indebted for permission to publish this account, we would wish especially to acknowledge the interest and assistance of Dr. Mary Cunningham. The influence of Dr. H. E. Williams in re-energising this inquiry has already been acknowledged, while the independent development of work along similar lines by Dr. S. C. Harland and Mr. Denham, at the Shirley Institute, has been of indirect assistance.

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* W. L. B., "The Existence of Daily Growth Rings in the Cell-wall of Cotton Hairs," *loc. cit.*

DESCRIPTION OF PLATE.

Figs. 1 to 7 inclusive, are all photographed with Spencer 4 mm. objective and 10 × eyepiece.

Fig. 1.—Transverse section of Sakel hair, possibly accidentally pressed, and slightly swollen with sub-critical strength sulphuric acid ; showing radial (spiral) cracks.

Fig. 2.—Transverse section of same cotton as fig. 1. Section lightly pressed, then swollen with critical strength sulphuric acid ; showing growth rings.

Fig. 3.—Ordinary Sakel hair mounted in liquid paraffin, untreated. (Denham and Harland's method.)

Fig. 4.—Hair of Sakel cotton boiled in dilute HCl, and pressed.

Fig. 5.—Hair of Sakel cotton pressed heavily in 8 per cent. caustic soda.

Fig. 6.—Hair pressed in naphthamine blue. Spiral structure entirely due to pressure only.

Fig. 7.—Hair of Sakel cotton pressed lightly in an adjusted mixture of cuprammonium and soda.

Figs. 8 and 9 are photographed with Spencer 16 mm. objective only, and 10 × eyepiece.

Fig. 8.—Hair swollen with calcium thiocyanate, showing double thread spiral.

Fig. 9.—Hair of Sakel cotton stained with iodine, but not otherwise treated ; showing double pit spirals.

Observations on the Distribution of Fat-Soluble Vitamines in Marine Animals and Plants.

By Johan Hjort, D.Sc., For.Mem.R.S.

(Received April 27, 1922.)

(From the Biochemical Laboratory, Cambridge.)

The Norwegian fishery investigators have for many years been engaged in the study of the growth of fish, mainly the herring and the cod. By means of microscopical study of the scales of the fish it has been possible to determine the age of each individual fish, and by means of the assumption, which has been verified within certain limits, that there is a proportion between the length of the scale (l_s) and the length of the fish (l_f) (*i.e.*, $l_s/l_f =$ constant), it has been possible to calculate the "growth curve" of the fish in different years of its life, and in different seasons of the year. The results of this work, which have been summarised up to the year 1914,* proved that the growth of the said fish in the Norwegian waters was confined to a few spring—and summer—months only, and that the growth of the fish entirely ceases during

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* Johan Hjort, "Fluctuations in the Great Fisheries of Northern Europe," 'Rapports et Procès-verbaux du Conseil International,' vol. 20, Copenhagen (1914).

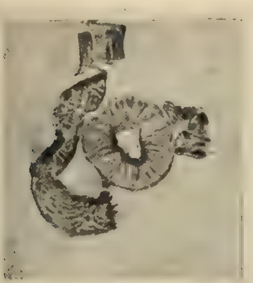

Fig. 1.

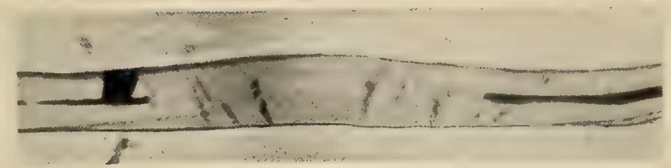

Fig. 3.

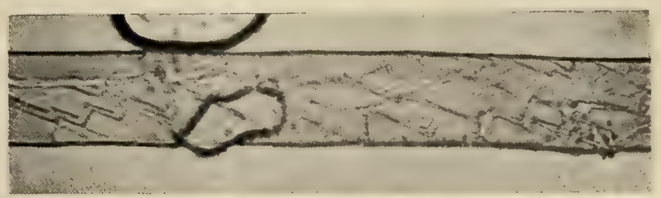

Fig. 4.

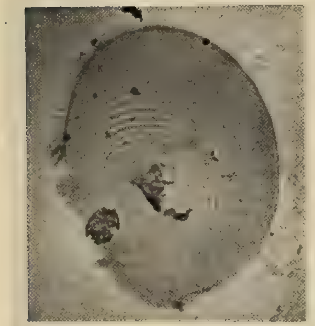

Fig. 2.

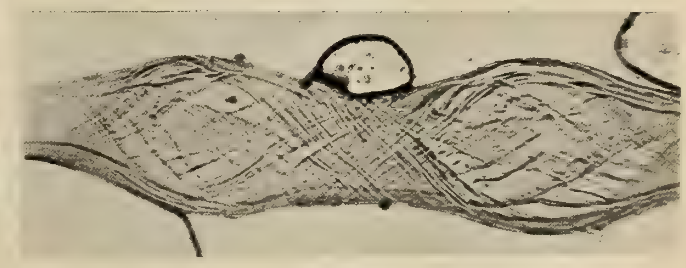

Fig. 5.

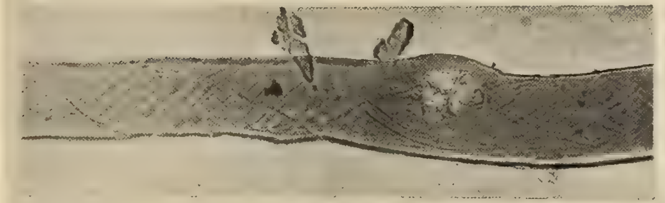

Fig. 6.

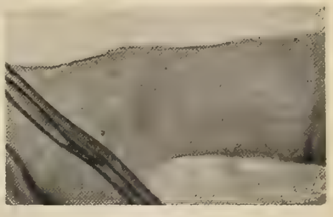

Fig. 8.

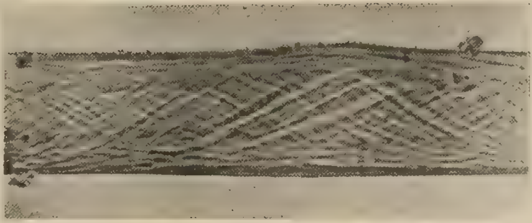

Fig. 7.

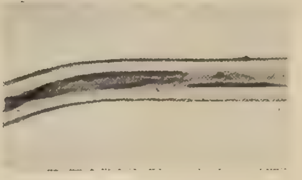

Fig. 9.

the winter season. Corresponding to the summer and winter zones, which are to be seen on the scales of the fish, we find a periodicity in the increase of length and weight of the fish, and a change in the quality of the fish at the different seasons of the year.

The changes in the "quality" of the fish were very early observed to be associated with the changes occurring in the content of fat. Chemical analyses were made by Mr. H. Bull of the fat contents of herrings and sprats at different months of the year,* and from these observations the conclusion was drawn that "the supply of fat increases during the summer and is consumed during the winter, while water is excreted in the summer and assimilated in winter. During the winter, part of the dry matter in the system is consumed and replaced by water, so that no great loss in weight is apparent. The quality of the fish, however, is considerably affected."

In the herring this is very apparent, the fat being deposited in a special peritoneal fat organ (by Norwegian fishermen called the "ister"), which is especially developed in summer and in the young year classes which have not yet developed their generative organs. As stated in the paper, the contents of the fat organ "varies with the age of the fish, the development of the genital organs, in particular, being a factor of great significance. In addition to this, the quality of ister varies greatly according to the time of year; this applies not only to the ister itself, but also to the contents of fat on the whole as shown by chemical analysis."

In the cod the changes in quality are most easily demonstrated by the inspection of the size of the liver, the contents of which, so far as some 50 per cent. is concerned, consist of the "cod-liver oil." Besides by chemical analyses, the liver has been studied by means of weighings. The livers of cod of different sizes (in groups of different lengths) have been weighed at different times of the year, and it has been found that the liver of the full-grown cod during the summer season, when the fish was feeding, weighed no less than three times as much as in the winter during the spawning season. "The greatest 'depreciation' in the quality of the cod takes place during their stay on the Lofoten Banks, where the genital organs arrive at full maturity and spawning takes place."†

These observations naturally led to a consideration of the problem of the conditions which determine the great changes in the growth of the fish themselves, and particularly in the organs in which the reserve material (fats) are stored. A comparison between the amount of fat in the sprat of the west coast and the mean surface temperature of the sea for the different months of the year* showed a remarkable correspondence for the spring and early summer months, *i.e.*, the amount of fat increases in close correspondence with the rise in temperature in the spring, but this relationship does not continue during the late summer and autumn, thus indicating that the temperature cannot be considered as the only influence concerned.

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* See *loc. cit.*, Chapter V.

† *Loc. cit.*, p. 195.

The rise of temperature in the sea during the months of spring is of course in northern waters accompanied with a great number of other phenomena, viz., the increase of the intensity of light, the increased addition to the sea-water of masses of fresh water from the continents carrying with them inorganic and organic substances from the land and the seashore. As a result of these events an "eruptive" development of plant life takes place, and shortly afterwards plankton animals (*i.e.*, copepods) develop and bottom animals begin to increase their growth.

While the development of the plants and of the small animals which directly prey on them may seem easy to understand as a result of the seasonal changes of the spring, the increase of the growth of larger animals like the cod, the prey of which exist all the year round, seems much more difficult to explain. The problem, therefore, seemed worth examination, whether the decrease of growth in the autumn and the increase of growth in the spring were connected with the availability of certain specific kinds of food, in other words if there could be found in nature a variation of chemical qualities in the food of the animals.

The fact that the changes in the quality of the animals during the different seasons have been found to be in such a close connection with changes in their contents of fats raised the desirability of investigating the distribution of the fat-soluble vitamines in organisms of the sea, since these vitamines have been found to have such a great influence on the growth of animals.

During my work at the Biochemical Laboratory of the University of Cambridge, Prof. F. G. Hopkins had the great kindness to offer me the opportunity of conducting experiments on such vitamine problems, and allowed me for this purpose to have the benefit of the organisation for experiments on rats which he has established at his laboratory. For the purpose of such experiments during the summer 1921 I made a collection of different material of plankton and bottom animals from Norwegian waters. This material was preserved in alcohol, and it was extracted in the laboratory (under anaërobic conditions) with alcohol and ether, in order to extract the oils for the experiments. This plan, however, had no success. The fats obtained in this way were in the form of waxes, and it was soon realised that for some reason they were unsatisfactory for nutritional experiments and gave ambiguous results. Other methods had therefore to be applied.

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* *Loc. cit.*, p. 173.

In the spring of 1922 material was, therefore, collected partly in a fresh or sterilized condition, partly in alcohol. In co-operation with my assistant, Dr. Axel Palmgren, a different method was developed for the extraction of the oils.

The fresh material was first minced, then dried in thin layers on filter paper in the constant-temperature room of the laboratory (at 37°). The dried substance proved in all experiments as effective as fresh substance, and it could therefore be applied for the purpose of extraction by acetone or benzene. The filtrates (from three to four extractions) were evaporated *in vacuo*, the last part of the solvent evaporated off in a water bath at 90°–100° in a high vacuum for 15 minutes, till the remaining oil no longer smelt of benzene. The oil was then dissolved in olive oil, and the mixture given to rats by a counted number of drops, 100 drops making 3 c.c. of oil. At a specific gravity of 0·9, one drop would then have a weight of 2·7/100, or 27 mgrm. If one part of the oil had been dissolved in seven parts of olive oil, eight or sixteen drops were given to each rat daily, in order to give the rat one or two drops of the oil under experiment. In the following "one drop" means one drop of the oil under experiment. The oil was not mixed with the food, but administered by means of a pipette kept just over the mouth of the rat, which was laid on its back in the left hand. This method of feeding was recommended to me by Prof. E. Poulsson, of the University of Christiania.

The experiments are all of a preliminary character, and had mainly the purpose of exploring the distribution of fat-soluble vitamines, for future more detailed and more quantitative studies. In spite of their preliminary character, it has been thought useful to publish the following observations, as they may stimulate other workers to investigate this field.

Marine Plants.

As a preliminary investigation, it seemed to me of interest to try some green algæ as representatives of the vegetation along the shore and some samples of the first growth of diatoms in the coast waters during spring.

Green algæ (*Ulva lactuca* and *Codium tomentosum*) were kindly collected for me at the Marine Biological Station at Port Erin by H. H. Thomas and S. H. Wadham, of the Botany School of Cambridge. The algæ were brought to us in fresh condition, and given partly in fresh and partly in dried condition to rats which for some time had been on a diet free from fat-soluble vitamines, but containing vitamine B. and other essentials. The three curves (figs. 1 and 2*) all show a marked increase in the weight of the rats when given the seaweed. Fig. 1, the lower curve, represents the growth on fresh seaweed, kept in cold store. The increase is not so marked as the two other curves showing the effect of dried seaweed. This produced an enormous increase in the weight. The dried seaweed was added to the food as a fine green powder and was thoroughly mixed with the food.

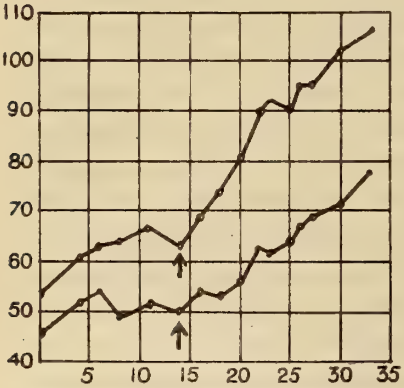

Fig. 1.—Lower curve, fresh ulva, *ad lib.* Upper curve, dried ulva, 1 grm. per day.

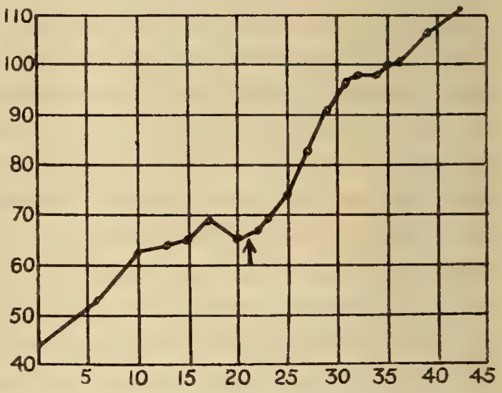

Fig. 2.—Dried ulva, 1 grm. per day.

In order to see if the growth-promoting factors of the ulva were soluble in its fats, dried powder of ulva was extracted with acetone and the oil given (1 or 2 drops a day) to the rats. The resulting growth-curves may be seen

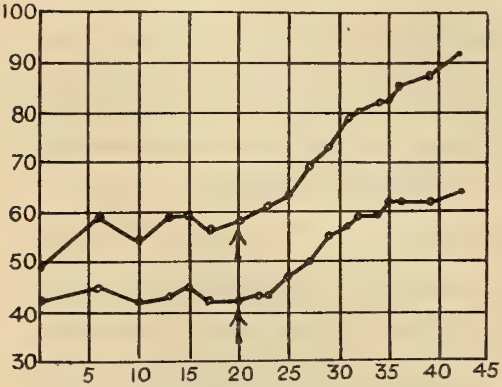

Fig. 3.—Extract (by acetone) of ulva, two drops a day.

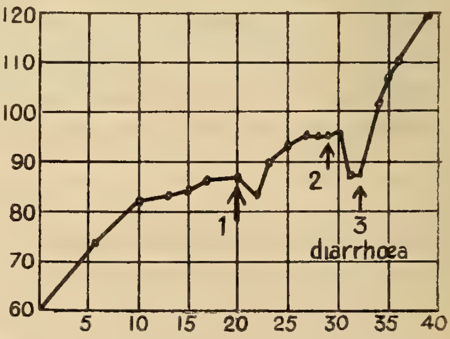

Fig. 4.—Point 1, added 1 gr. sterilised diatoms per day. Point 2, 1·5 grm. dried diatoms per day. Point 3, 2 drops benzene extract of diatoms per day.

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* In all the figures the abscissa gives the number of days of experiment, the ordinate the weight in grams.

from fig. 3. They demonstrate a marked increase in growth of the rats from the moment when the oil was given.

Samples of *Diatoms* were collected for me by my friend, Prof. H. H. Gran, and Mr. E. Lea on board a Norwegian research vessel. The samples consisted of a pure "Diatom-plankton," as this may be found in the sea in the spring before the animals have started their development. The rats fed on these diatoms showed first a sharp increase of growth, and then, after a short time, a marked decline. The idea suggested itself that the diatoms, which contain so much silica, may be very unsuitable as food for a mammal, and diarrhœa was also observed in one case (see fig. 4). In some cases, two drops a day of green oil, extracted by benzene from the dried mass of diatoms, gave at once a marked increase, even in the case of animals which had first been fed on the dried material (fig. 5). Although these experiments, which were hampered by rather scanty material, do not give such a marked and striking growth as the curves of the seaweed, I have no doubt that an increased growth has been demonstrated as a result of addition to the basal diet of the rats of oils extracted from diatoms.

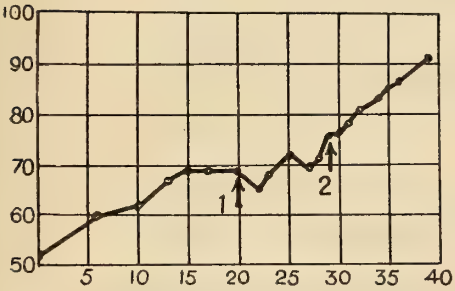

FIG. 5.—Point 1, *ca.* 0·8 grm. dried diatoms per day. Point 2, 2 drops extract per day.

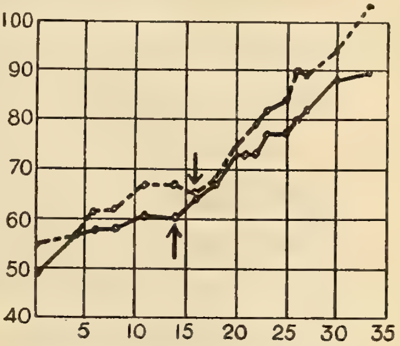

FIG. 6.—Fresh shrimps *ad lib.*

Shrimps and Prawns.

Fresh shrimps (*Crangon*) and fresh prawns (*Pandalus borealis*) were minced and given fresh as an addition to the basal diet of several rats. Figs. 6 and 7 show a marked increase in the weight of the rats shortly after the addition of these substances to their diet.

Dried powder was extracted by benzene, the extracted oil (two drops a day) had in several cases no effect, a fact which I am not able to explain and which must be left for future investigation.

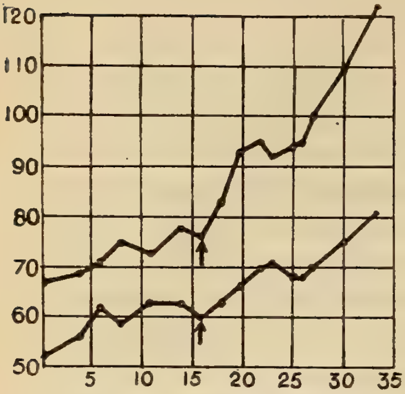

Fig. 7.—Fresh prawns *ad lib.*

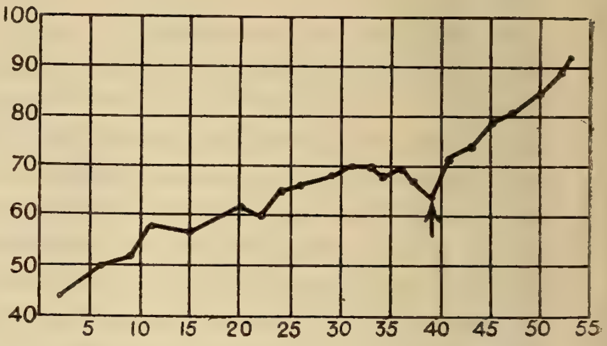

Fig. 8.—Four drops of oil from roe of herrings.

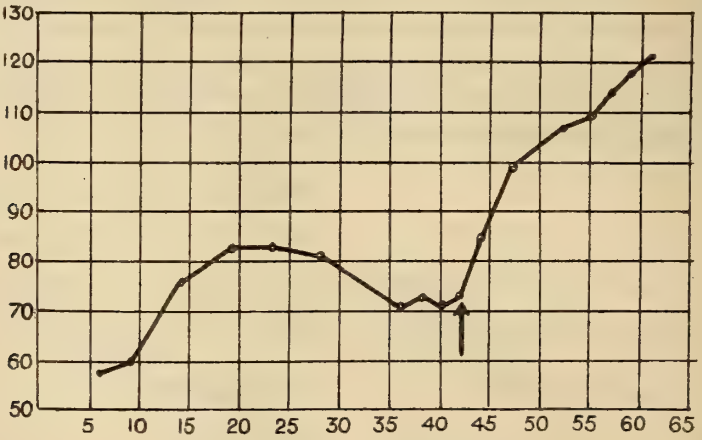

Fig. 9.—Fresh cod roe *ad lib.*

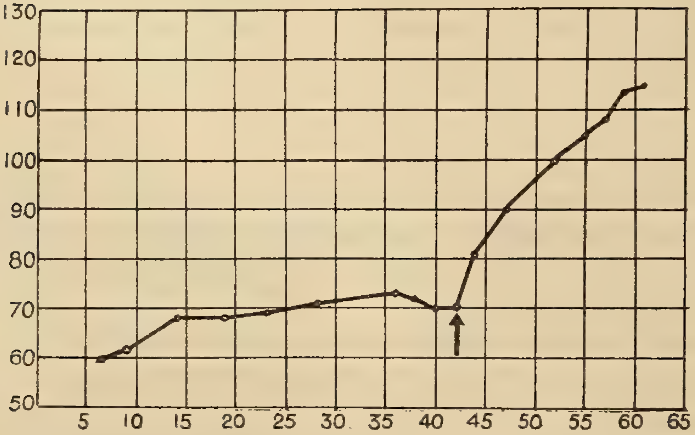

Fig. 10.—Fresh cod roe, 2 grm. per day.

Herring and Cod.

I have mentioned in the introductory remarks above that the fat organ of the herring and the liver of the cod both decrease in weight at the same time as the ovaries of the female animal increase in weight. An examination of the contents of fats and of fat-soluble vitamines of the ovaries (the roe) would therefore seem desirable. Fig. 8 shows the effect of addition of oil extracted from the ovaries of herrings.

Fresh cod roe (figs. 9 and 10) in a dose of 2–3 grm. per day produced an immediate and rapid increase in the weight of rats. Dried roe, prepared in Norway for commercial purposes (1 grm. added per day), had (figs. 11 and 12) just as marked an influence; and this fact must, like the effect of the dried ulva, be considered as very important, since it proves that the process of drying fish products had no observable destroying effect on the growth-

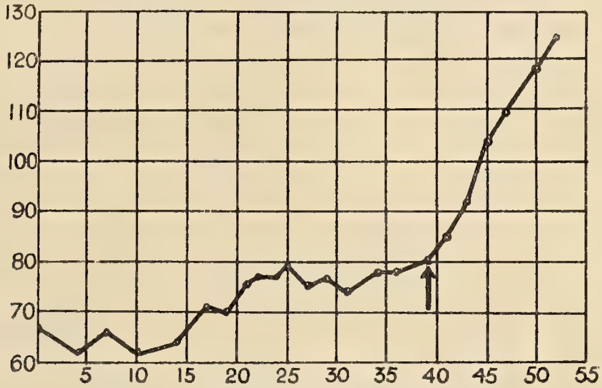

Fig. 11.—Dried cod roe, 1 grm. per day.

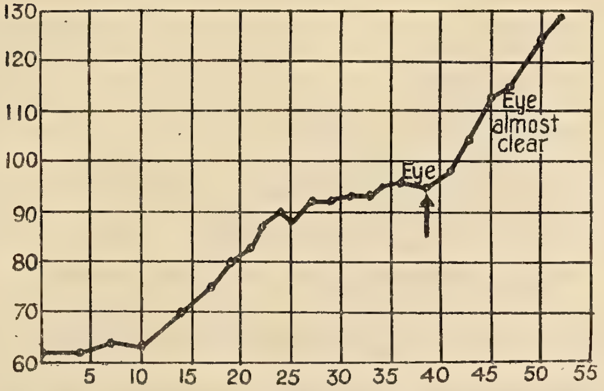

Fig. 12.—Dried cod roe, 1 grm. per day. When first given one eye very bad, after some days almost clear.

promoting factors. Drying of fish and fish products plays a great *rôle* in the fishing industry of different countries, and it is therefore of practical value to know that the process does not deprive the products of these valuable qualities.

Extraction of the powdered dry roe by means of benzene produced a clear but thick oil, different from the wax, which was obtained by extraction with alcohol and ether. The effect of one drop daily of this oil is illustrated by the curves on fig. 13. In one rat, which had developed the eye disease (keratomalacia) characteristic of cases upon a diet deficient in the fat-soluble vitamine, this disease was cured after the addition of the oil of the roe, but this rat did not later on show any increase in its growth.

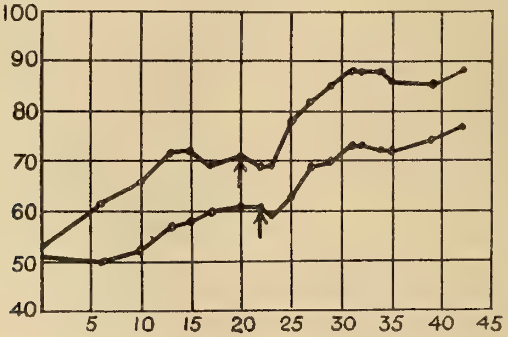

Fig. 13.—Benzene extract of dried cod roe, 1 drop per day.

The fact that oils extracted from marine plants have been proved to have a very strong effect on the growth of rats fed on a diet deficient in fat-soluble vitamines seems to indicate the working hypothesis that fat-soluble vitamines in the sea arise in plants in a similar way to that proved to be the case in plants on land. It seems, further, natural to assume that all marine animals, directly or indirectly, obtain these substances from the plants, and that the marine animals, like other animals studied, are unable to synthesise these substances. An important programme for future work seems then to suggest itself, viz., to determine the quantitative distribution of fat-soluble vitamines in different types, and in them during different seasons of the year. It may be possible by such studies to approach the important problems of the influence of the food of animals on that periodicity in their growth which is so remarkable in Northern waters. The present investigations seem to indicate the problem to ascertain how far the production of fats and fat-soluble vitamines in the plants during spring and early summer months will have to be regarded as the store on the magnitude of which the growth of animals is dependent in Northern latitudes. In Southern latitudes the

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BIOLOGICAL SCIENCES

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growth of fish has been found much more uniform throughout the year; the changes in the production of plants are there not so marked as in the high Northern latitudes.

Dr. Palmgren and I intend, if circumstances permit, to continue the investigations towards the solution of these problems at the Biological Laboratory of the University of Christiania.

On Blood-Platelets: their Behaviour in "Vitamin A" Deficiency and after "Radiation," and their Relation to Bacterial Infections.

By W. Cramer, A. H. Drew, and J. C. Mottram.

(Communicated by Prof. W. Bulloch, F.R.S. Received March 28, 1922.)

(From the Laboratories of the Imperial Cancer Research Fund and from the Radium Institute.)

Introductory. General Effects of Fat-soluble Vitamin Deficiency.

When the fat-soluble vitamin A is withheld from the diet of a rat, the general condition of the animal differs from that resulting from a deficiency of the water-soluble B vitamin. In the latter case the animal ceases to increase in weight almost at once, and then begins to decline. There is a progressive fall in the temperature. The animals always die within a comparatively short time—two months—and are then found to be in a state of profound emaciation, as if they had received no food at all. There is no obvious sign of disease or of an infection. For the sake of convenience, we will designate briefly this condition by the term "marasmus."

The withholding of the fat-soluble vitamin A alone affects different individual rats in a manner as varied and indefinite as the conditions obtained by a deficiency in the water-soluble B are constant and definite. When a young rat is kept on a diet from which the fat-soluble vitamin A is absent, the increase in weight may cease almost at once, or it may continue to increase in weight for many weeks, and almost as rapidly as on a diet containing vitamin A, although eventually its growth will come to a standstill before the full normal size of an adult rat has been reached. We shall, for the sake of convenience, describe these two extremes as the "acute" and the "chronic" effect on growth respectively. Eventually the rats develop infective conditions, which attack most frequently the eyes, and xerophthalmia develops. There may be other organs affected (septic glands or pneumonia sometimes develop).

These infective conditions develop most rapidly in those rats which show the "acute" effect on growth, when it may first appear after 6–8 weeks. In the rats showing only the "chronic" effect on growth, an infection may not develop at all or very much later. We have kept rats on a fat-soluble vitamin-free diet for four months without any infection appearing, although these rats were kept in the same cage with rats which had developed a very intense xerophthalmia. The external appearance of these rats was, in fact, such as to make them indistinguishable from normal rats. It will be shown, however, that such rats develop the lesion which we consider to be specific for the vitamin A deficiency, although not to the same extent as rats exhibiting the acute effect on growth. The condition of nutrition of rats on a vitamin A-free diet varies, as is to be expected. The rats which show the acute condition are ill-nourished, but the extreme condition of emaciation seen in the rats suffering from the B deficiency is hardly ever met with. Another important difference is that the progressive fall of temperature, which is so characteristic of the B deficiency, does not appear in the A deficiency.

We have been able to control experimentally the conditions under which the "acute" and "chronic" effects of fat-soluble vitamin deficiency appear. Hitherto, the reason for these differences has not been understood. One important factor is the nature of the diet which the rats have received in infancy. If the mother during pregnancy and lactation has received a diet rich in vitamins, and if this diet be given to the young rats after they have been weaned, then the rats are found to be more resistant to a subsequent withdrawal of the fat-soluble vitamin. If, on the other hand, the diet has not been particularly rich in vitamins, although it may contain an amount adequate to maintain the animals in health and to enable them to grow and to breed freely, then withdrawal of the fat-soluble vitamin will produce an immediate and "acute" effect. It may be added that the diet we refer to is not an artificial one, but a natural diet of bread and water, rice and maize, which has for years been used in our laboratory as the standard diet for our stock of rats. Incidentally, this clearly emphasises the great importance of assuring an ample supply of vitamins to the pregnant and lactating woman, and to children, and of not being satisfied with the comfortable belief that our ordinary food contains sufficient vitamins, because we do not suffer from deficiency diseases.

Another factor which determines the onset of xerophthalmia is the amount of the water-soluble vitamin supplied. When a small amount of this vitamin is given, xerophthalmia develops much earlier than when a large amount is given. This statement refers to experiments in which the water-soluble vitamin was supplied in the form of "marmite."

Between the two extremes which we have described, all intermediate conditions may be observed. These large variations in reaction to a withdrawal of the fat-soluble vitamin make it, of course, very difficult to appraise the significance of any changes in tissues and organs which may be observed. Many changes which can be seen and have been described must be regarded as accidental. For instance, the failure in nutrition which may occur is obviously not an essential feature, and is not necessary for the onset of infective conditions. In connection with our previous work on the relation between lymphoid tissue and nutrition, it is of interest to note that there may be a considerable lymphopenia when the animals have shown the "acute" effect and are in a poor state of nutrition; but when the eye condition develops in a well nourished animal, the lymphoid tissue is normal and the lymphocyte count shows only a slight diminution. The only general feature common to all rats which have been subjected to a fat-soluble vitamin deficiency is a greatly diminished resistance to infection.

We have dealt in considerable detail with the great variety in the general conditions of animals on a vitamin A-free diet, because a lesion, if it is to be considered specific to this deficiency, must be present in all these animals. Further, the severity of such a lesion should be found to vary with the extent to which the animal is affected by this vitamin deficiency. And, lastly, the lesion should disappear when the deficient vitamin is supplied, and the animal recovers as the result.

We have found such a lesion in the great reduction in the number of blood-platelets. We were led to look for a change in the platelets, because we noticed an obvious change in the condition of the blood of rats kept on a diet deficient in the fat-soluble vitamin. When the tail was cut for the purpose of examining the red and white corpuscles, the blood flowed much more freely than in a normal animal, and it was much more difficult to arrest the bleeding. When a film was made, it was more difficult to obtain an even spreading. There were no constant differences in the number of white corpuscles or of the red corpuscles to account for this change, although, as will be seen later, there may be, in advanced stages of the deficiency, a distinct anæmia. But the diminished coagulability of the blood, as it manifests itself by the difficulty of arresting the bleeding, sets in long before the anæmia occurs. The essential importance of the platelets in blood coagulation, which the previous observations of Cramer and Pringle (1) and of Bordet (2) had demonstrated, led us to examine the blood for platelets.

Method of Counting Platelets in Rats.—The animal is deeply etherised, and the tail placed in a watch-glass filled with a solution of 2 per cent. sodium citrate in 0·6 per cent. NaCl solution. After cutting the tail and allowing the blood to flow until free bleeding is established, the tail is transferred quickly to another watch-glass containing about 1 c.c. of the citrate solution or of Toisson's fluid. Blood is allowed to flow so that a mixture of the solution and blood convenient for counting the red blood corpuscles and the platelets is obtained. After the first few counts it is easy to recognise when the mixture is of convenient concentration. The tail is then wiped dry and the pipette of the hæmocytometer is then filled with blood in the usual way, so as to obtain a count of the absolute number of red corpuscles. In order to count the number of platelets, the citrate-blood mixture is thoroughly stirred, a standard drop placed on a large slide and covered with a cover-glass, which is then cemented with melted paraffin. After allowing the cells to settle, the proportion of red cells to platelets in each field is counted, until about thirty platelets have been counted. From this the absolute number of platelets can be calculated. A helpful device for counting the platelets, which has proved very useful, is as follows: A coarse grating of about 1 mm. squares is ruled on a piece of ground glass with a lead pencil. This is mounted in balsam under a cover-glass, and placed close up to the source of light at right angles to the beam. The image of the grating is then focussed by means of the substage condenser, after the preparation has been focussed with the objective, and conveniently divides the field for counting.

Red Cells and Platelets of normal Rats.—The following Table gives these data for nine normal rats. The counts for the first five rats were made in one laboratory, those of the last four in the other. The figures obtained show

Table I.—Normal Rats.

Weight in grm.	Red cells.	Platelets.
150	10,400,000	960,000
150	11,840,000	730,000
150	10,660,000	845,000
100	10,480,000	720,000
120	9,960,000	660,000
50	8,194,000	786,000
50	10,928,000	1,050,000
60	10,128,000	912,000
60	9,888,000	1,000,000

that the average red cell count for the normal rat lies approximately between 9,000,000 and 10,000,000 cells per cubic millimetre, the average platelet count lies approximately between 700,000 and 900,000 per cubic millimetre. The variations which have been found include not only individual differences of different rats, but also differences due to age, feeding, and those due to the personal factor involved in the technique. The various counts made on rats showing complete recovery from the vitamin A deficiency (see fig. 4 and Table) and from the effects of radium (see fig. 5) give similar figures.

Effect of Vitamin A Deficiency on Platelets.—The rats were kept on a basal ration of casein, starch, autoclaved olive oil and the usual salt-mixture, to which vitamin B was added in the form of marmite. The olive oil prepared as above was known to be free from vitamin A. The casein was freed from the fat-soluble vitamin in some experiments by repeated extraction with alcohol and ether, in others by heating in shallow trays for 24 hours to 130° in air. On such a diet free from the fat-soluble vitamin the platelets show a progressive diminution in their number, and this "thrombopenia"—as it may be called—proceeds *pari passu* with the decline in the general condition of the animal. Thus in the one extreme condition, when the weight of the animal becomes stationary directly the vitamin A is withheld, and when eye symptoms develop within two months, the fall in the number of platelets is rapid and pronounced. Taking the opposite extreme, when the animal continues to grow at an almost normal rate for several weeks, and infective conditions do not make their appearance until much later or not at all, then the fall in the number of platelets is delayed and less pronounced, though still distinct. In fact, a slight fall in the number of platelets may sometimes be the only sign of the vitamin A deficiency, the rat looking quite normal and healthy, and having perhaps only a slightly subnormal weight. Our observations show that infective conditions (xerophthalmia, etc.) do not develop until the platelets have fallen below about 300,000 per cubic millimetre.

It is important to note that the onset of these infective conditions depends on the level to which the platelets have fallen, and not on the length of time to which the rats have suffered from a vitamin A deficiency, nor on exposure to infection, as will be shown presently. When the deficient vitamin A is again supplied after a low platelet count has been established, the number of platelets increases. Here again there is a close parallelism between the rate of increase in the number of platelets and the degree of improvement in the animal.

These statements are based on and illustrated by the experimental data given in the following figures and Tables, which explain themselves and need little further general comment. In the weight curve of each rat the arrow indicates a count, and the number attached to the arrow gives the number of platelets in thousands. Thus "217" means 217,000 platelets per cubic millimetre. The onset of eye symptoms is indicated by "*x*." More advanced stages are indicated by "*xx*" and "*xxx*." In the figures which illustrate the recovery from the vitamin A deficiency, the broken line represents the weight curve during the last weeks of the absence of the vitamin, the full line gives the weight curve after the addition of this vitamin in the form of cod-liver oil. Most of these recovery curves refer to animals dealt with in the preceding figures, as will be evident from the rat numbers attached to each curve. In these recovery curves the sign ⓧ stands for the complete disappearance of the xerophthalmia.

The figures illustrate all the different varieties of conditions which can be observed in rats when kept on a diet deficient in vitamin A. Special attention is drawn to fig. 1, which refers to an experiment specially devised to illustrate the two extreme conditions and the parallelism between the effect of the vitamin deficiency on the general condition of the animals and on the platelets.

In this experiment two groups of three rats derived from two litters, X and Y, were taken. The litters were born within three days of each other. The mother of the litter X had been kept during pregnancy and lactation on the ordinary laboratory diet of bread and water, rice and maize. This diet was continued for the young rats after they had been weaned until the actual experiment began. The mother of the other litter, Y, had been kept on the same diet, to which an ample supply of vitamins A and B had been added in the form of cod-liver oil and marmite, and this diet also was continued for the young rats.

When the rats were 7 weeks old, the three heaviest and healthy-looking of each litter were selected, and the six rats placed together in one cage and fed with the vitamen-free basal ration (purified casein, starch, olive oil, salt mixture), to which an ample supply of vitamin B was added in the form of crude marmite. As fig. 1 shows, the rats derived from litter X stopped growing almost at once and developed the typical eye infection within 8 or 9 weeks. Those of litter Y continued to grow fairly well at first. After the eleventh week their weight became stationary and remained so for the next 6 weeks (at the time of writing). Up to that time they had not developed any lesion or other infective conditions. They looked perfectly healthy normal rats in a good state of nutrition, and formed a striking contrast with the small, thin, infected rats of litter X. It may be added that the same result was previously obtained in a similar experiment, while in a third such

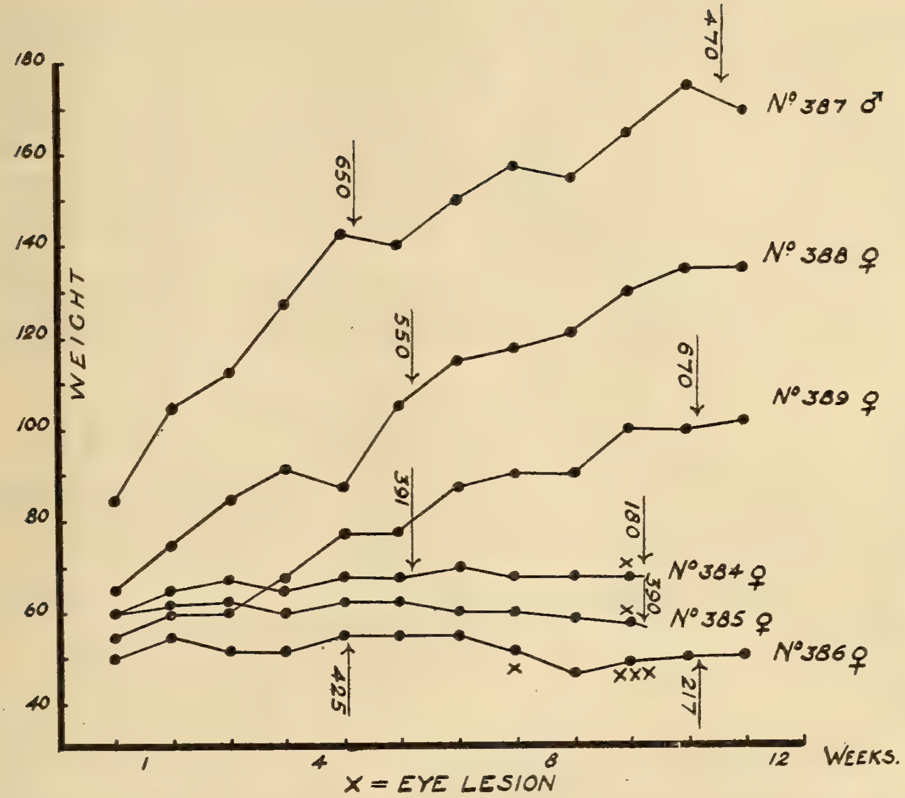

FIG. 1.—Weight curves and platelet count in six rats kept in the same cage on the same vitamin A-free diet. The figure illustrates the parallelism between the degree of thrombopenia and the general condition of the animals.

Table to Fig. 1.

No. of rat.	Weeks of A deficiency.	Red cells.	Platelets.	Condition of rat.
386	4	8,720,000	425,000	No growth.
387	4	7,450,000	650,000	Growing rapidly.
384	5	8,320,000	391,000	No growth.
388	5	8,400,000	550,000	Growing.
384	9	6,200,000	180,000	No growth, thin. Xerophthalmia beginning.
385	9	9,320,000	390,000	
386	10	8,120,000	217,000	Looks very ill. Advanced xerophthalmia.
387	10	9,880,000	470,000	Normal healthy appearance.
389	10	8,800,000	670,000	

experiment the difference, although still present, was not so striking. In the present experiment the blood was examined at different times after the withdrawal of vitamin A in such a way that the number of platelets in a rat of one group could be compared with that of a rat of the other group on the same day. The results, which are arranged in this way in the Table to fig. 1, show clearly that both groups develop a progressive thrombopenia, but that this thrombopenia advances much more rapidly in the severely affected litter X than in litter Y.

Fig. 2 refers to four rats which react in the usual way to the withdrawal of the fat soluble vitamin from the diet, and requires no further explanation.

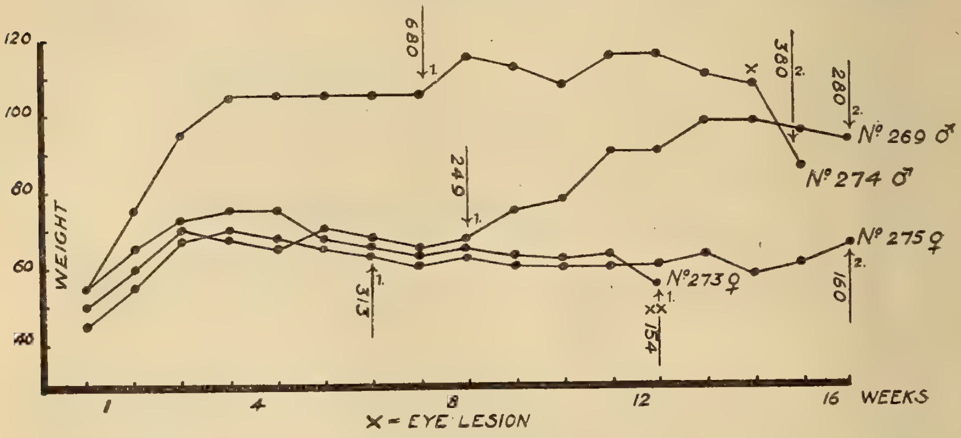

Fig. 2.—Weight curves and platelet count of four rats kept on a vitamin A-free diet.

Table to Fig. 2.

No. of rat.	Weeks of A deficiency.	Red cells.	Platelets.	Condition of rat.
269	8 16	8,720,000 8,240,000	249,000 280,000	No eye symptoms.
273	12	6,720,000	154,000	Intense xerophthalmia. Bacteria in blood.
274	7 15	10,760,000 10,320,000	680,000 380,000	Xerophthalmia.
275	6 16	8,000,000 7,800,000	313,000 160,000	Eye symptoms developed two weeks after this count.

We have stated above that, when A is withheld, the amount of vitamin B supplied determines to a certain extent the onset of the typical symptoms.

Fig. 3 refers to an experiment on three rats in which a minimal amount of vitamin B was supplied. The amount given was sufficient to prevent a fall of temperature, but was not sufficient to enable the animals to grow. Two of the three animals, Nos. 398 and 399, received no vitamin A, and they rapidly developed the typical eye lesion, although their weight was over 100 grm. when the experiment began. A third animal, No. 397, received an ample supply of the vitamin A, and served as control. It did not grow, but remained in good health otherwise. In this rat the number of platelets remained normal, while the other two rats developed an intense thrombopenia. Further evidence that the absence of vitamin B does not markedly affect the platelets will be given below.

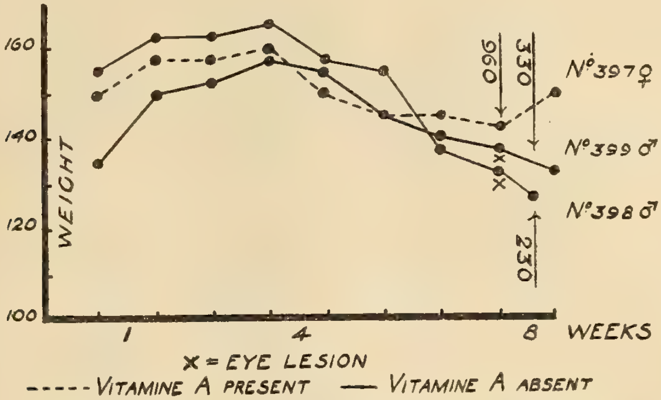

Fig. 3.—Effect of restricting the water-soluble vitamin B to a minimum. Two rats, Nos. 398 and 399, received no fat soluble vitamin, and developed the typical eye lesion. The third rat, No. 397, received an abundant supply of vitamin A, and remained well.

Table to Fig. 3.

No. of Rat.	Weeks of experiment.	Vitamins supplied.	Red cells.	Platelets.	Condition of rat.
398	7	A absent, minimal supply of B	9,680,000	230,000	Xerophthalmia ; died two days later of pneumonia.
399	7		9,400,000	330,000	Xerophthalmia.
397 Control	7	Ample supply of A, minimal supply of B.	10,400,000	960,000	Has not grown, but healthy appearance.

Perhaps the most striking evidence of the relationship between the fat-soluble vitamin and the platelets, is afforded by the behaviour of platelets when the animals recover from vitamin A deficiency. This is illustrated in fig. 4. When the fat-soluble vitamin is supplied again, the platelets increase with extraordinary rapidity. Here, again, there is a close parallelism between the recovery from the eye lesion and the increase in the number of platelets. Thus, in rat 277, the eye lesion completely cleared up within a week, and the number of platelets increased from 380,000 to 870,000. In rat 250, with the low value of 220,000 platelets and the presence of bacteria in the blood as demonstrated by a film preparation, the platelets increased to almost 1,000,000 within three weeks, the eye lesion became completely cured within two weeks, and the bacteria disappeared from the blood. The reverse condition is represented by rat 386, which was very small and thin and emaciated, and which, in addition to having the eye lesion, developed an abscess in the neck. When cod-liver oil was given the eye lesion cleared up very slowly, and the general nutritive condition of the animal did not improve very much; as the

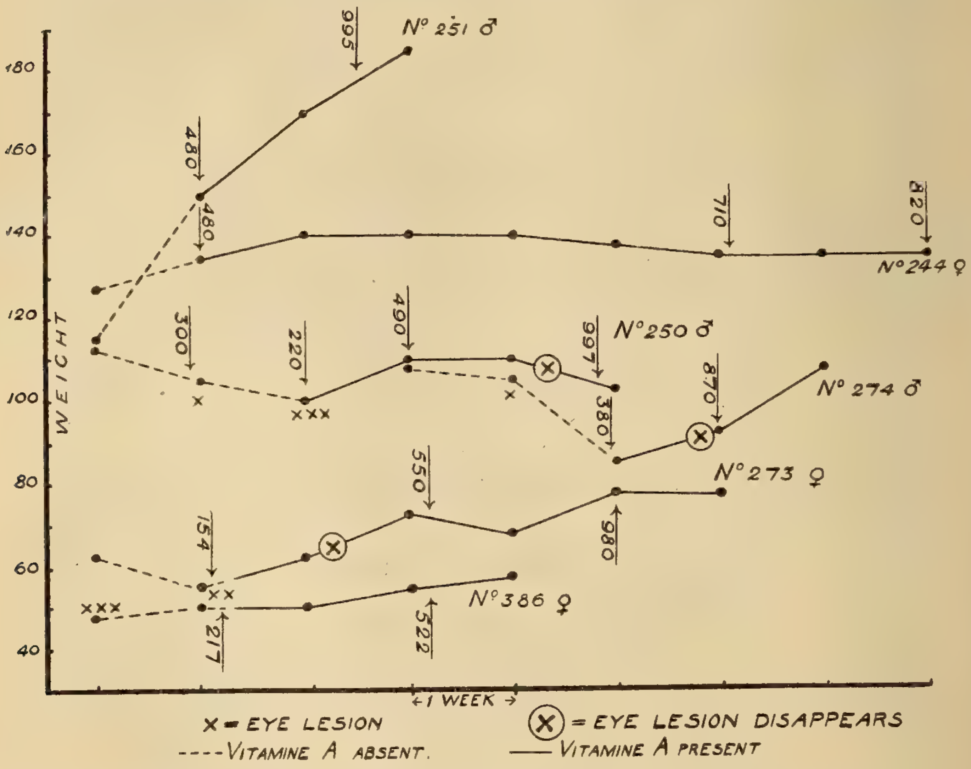

FIG. 4.—Effect of adding vitamin A to the deficient diet. The figure illustrates the parallelism between the rate of increase in the number of platelets and the recovery from infection.

Table to Fig. 4.

No. of rat.	Deficiency or recovery.	Red cells.	Platelets.	Condition of rat.
244	Deficiency	8,720,000	480,000	
	Recovery, 5 weeks	10,440,000	710,000	
	7 weeks	10,000,000	820,000	
250	Deficiency	9,280,000	300,000	Xerophthalmia, bacteria in blood.
	Deficiency	10,080,000	220,000	Xerophthalmia worse.
	Recovery, 1 week	8,000,000	490,000	Eyes improving.
	3 weeks	9,960,000	997,000	Cured.
251	Deficiency	7,560,000	480,000	
	Recovery, 2 weeks	8,360,000	995,000	
273	Deficiency	6,720,000	154,000	Xerophthalmia.
	Recovery, 2 weeks	9,720,000	550,000	Eyes cured.
	4 weeks	9,760,000	980,000	
274	Deficiency	10,320,000	380,000	Xerophthalmia.
	Recovery, 1 week	10,560,000	870,000	Eyes cured.
386	Deficiency	8,120,000	217,000	Intense xerophthalmia, abscess in neck.
	Recovery, 2 weeks	6,800,000	522,000	Looks thin and ill; xerophthalmia had almost cleared up; abscess still present, but improving.

weight curve also indicates. The platelet count showed only a slow rise from 217,000 to 522,000 after two weeks.

Effect of Vitamin A Deficiency on the Red Cells.—The Tables show that, in the majority of cases, there is no distinct reduction in the number of red cells, even when the platelets are greatly diminished and the animal is in a typical condition of A deficiency. Occasionally, however, an anæmia develops. Rats Nos. 384 and 273 have a distinct anæmia with a red count between 6,000,000 and 7,000,000. These animals also had the most profound thrombopenia. Rat 386 is of interest because it developed an anæmia, not while the vitamin A was withheld, but later on when it was supplied again. It will be recalled that this animal had suffered severely from the deficiency and responded with only an incomplete recovery. The anæmia cannot, therefore, be regarded as the characteristic lesion of vitamin A deficiency. Our observations indicate that these occasional anæmias may follow an infection of the blood with micro-organisms.

Effect of Vitamin A Deficiency on the Leucocytes.—This subject has already been dealt with in a previous paper. There are no constant or characteristic changes. In the final stage, when infection has supervened, there is usually a great increase in the absolute number of polymorphonuclear cells. The lymphocytes show, as a rule, no more than a slight diminution, which contrasts sharply with the profound lymphopenia observed when the water soluble vitamin is withheld. We have already stated that a lymphopenia may also occur in the vitamin A deficiency when the acute effect has been produced and the animals are in a very poor state of nutrition.

There appears to be a change in the number of the polymorphonuclear leucocytes, in the sense that the nucleus is less lobulated in the animals suffering from the vitamin A deficiency. To establish this fact fully would require a very extensive series of observations which we do not propose to undertake. We only refer to it here because it may afford an explanation of the statement that the so-called "Arneth index" (number of lobules of the polymorph nucleus) of tuberculous individuals is higher than that of normal individuals. The explanation may possibly be found in the fact that the high Arneth index of the tuberculous individual is due to his dietary treatment, the diet being very rich in the fat-soluble vitamin.

Effect of Vitamin B Deficiency.—Some observations were made on rats kept on a diet free from vitamin B, but containing an ample supply of vitamin A. The results which are given in the following Table show that when the vitamin B is absent and vitamin A present the platelets do not diminish to any extent, even at a time when the temperature has become very distinctly subnormal, and indicates an advanced stage of the deficiency :—

Effect of B Deficiency.

No. of rat.	Weeks of B deficiency.	Temperature.	Difference of initial weight.	Red cells.	Platelets.
		°			
414	5	35·7	0*g*	11,560,000	980,000
415	5	35·2	0*g*	9,920,000	640,000
416	5	Below 35	−5*g*	10,000,000	1,200,000

In the very last stages of this deficiency we have occasionally obtained low figures for the platelets. But here a technical difficulty arises, because it is difficult in that condition to obtain a free flow of blood when the tail is cut. This is, however, essential, since the blood platelets tend to stick to the tissues of the wound and disintegrate there, when the blood is oozing out slowly.

Even in a normal animal a low count is obtained if, for some reason, a free flow of blood cannot be established.

Effect of Malnutrition Not Due to Vitamin Deficiency.—In order to study this effect young rats of about 50 grm. weight were kept on a protein-free diet, consisting of starch, salt mixture, and olive oil, to which the vitamins A and B were added in the form of cod-liver oil and marmite. As a result the rats decline in weight, but remain otherwise in good health, for three or four weeks. The platelets show no diminution at a time when the animals have lost 10 grm., *i.e.*, 20 per cent. in weight, as the following figures show :—

Effect of Protein Deficiency in the Presence of Vitamins.

No. of rat.	Weeks of protein deficiency.	Temperature.	Loss in weight.	Red cells.	Platelets.
		°			
420	3	38·4	10*g*	9,776,000	1,060,000
421	3	38·3	10*g*	9,280,000	930,000
422	3	38·3	10*g*	10,370,000	1,230,000

Effects of Exposure to Radium.

It is well known that a profound lymphopenia can be produced and maintained by relatively small doses of β or γ radiation. With larger doses additional blood changes occur: first a diminution in the number of polymorphs and with still larger doses a reduction in the number of red cells and hæmoglobin content. For instance, when rats were continuously exposed to radium under constant conditions, a lymphopenia occurred within a few hours, a polymorpho-leucopenia in 7 days and an anæmia in 13 days.

Examples of these effects are given in Protocols Nos. 1 and 2.

Protocol No. 1 showing changes in the polymorphs:—

Three male rats: weights 150, 160, 155 grm., exposed continuously to 220 mgrm. $RaBr_2, 2H_2O$, distance 8 inches, screen 0·1 mm. lead, 0·12 mm. silver, for 7 days—this is equivalent to 0·55 rads.

Two control rats: weights 210 and 165 grm.

Protocol No. 2 showing changes in red cells and hæmoglobin. Three female rats: weights 80, 85, 75 grm. exposed, continuously to radium, as in Protocol No. 1, for 13 days, equivalent to 1·2 rads.

Three control female rats: weights 80, 85, 75 grm.

The following Table gives the results of the blood examinations :—

Rat.	Weight.	—	Days.						
			0.	2.	7.	11.	16.	23.	30.
Radium	160	P	4·0	2·2	1·5	1·9	1·4	3·0	4·3
		L	10·5	5·4	1·4	1·2	2·4	7·2	7·7
Control	210	P	9·2	7·6	7·4	8·3	5·2	—	—
		L	13·1	15·0	24·0	27·1	20·2	—	—
			0.	2.	4.	8.	14.	18.	25.
Radium	155	P	4·2	4·8	2·2	0·9	1·1	1·0	3·7
		L	20·6	10·2	3·1	1·2	3·4	9·6	10·1
Control	165	P	3·8	3·7	4·6	3·6	5·8	3·2	—
		L	14·7	15·6	23·2	20·2	17·1	20·3	—
			0.	3.	8.	15.	21.	28.	
Radium	150	P	5·3	3·5	1·4	2·6	2·3	3·7	
		L	15·1	4·9	2·0	7·4	9·6	7·4	

P = polymorphs. L = mono-nuclears in thousands per c.mm.

The following Table gives the results of the blood examinations :—

Rat.	Weight.		Days.				
			0.	6.	13.		
Radium	80	R	9·1	8·3	4·1		Dead on 17th day.
		H	102 p.c.	97 p.c.	53 p.c.		
Control	80	R	7·5	8·0	9·1		
		H	98 p.c.	—	105·5 p.c.		
			0.	9.	13.	18.	
Radium	85	R	9·2	7·5	8·0	4·9	Dead on 20th day.
		H	97 p.c.	91·5 p.c.	83 p.c.	69 p.c.	
Control	85	R	8·2	8·5	8·3	10·1	
		H	104·5 p.c.	111 p.c.	101·5 p.c.	115 p.c.	
			0.	13.			
Radium	75	R	7·6	2·1			Dead on 17th day.
		H	96·5 p.c.	—			
Control	75	R	8·5	10·3			
		H	108 p.c.	100 p.c.			

R = red cell content in millions. H = hæmoglobin percentage.

At death these animals exhibited signs of a generalised infection with micro-organisms accompanied by a bronchopneumonia or an enteritis; in one case xerophthalmia was present. If instead of continuing the exposure for 13 days, shorter exposures be given, then an anæmia will either not develop or will supervene after varying lengths of time according to the dose and the weight of the animal; and it is remarkable that so far whenever an animal has developed an anæmia it has invariably died within a few days. This suggests that the anæmia is not directly due to the radiation, but is a secondary effect possibly due to the invasion of the blood stream by micro-organisms. In view of the similarity between these effects and those described above as resulting from withholding vitamin A, an examination of the platelet content of the blood was made to discover whether this could be the primary change to which the invasion of the blood stream by micro-organisms and the anæmia was secondary. The findings are given in the following protocol.

Protocol No. 3.

Five rats: weights 60, 60, 65, 70, 90 grm., exposed as in Protocol No. 1 for 5 days, equivalent to 0·46 rads.

The red cell and hæmoglobin content remained normal in every case.

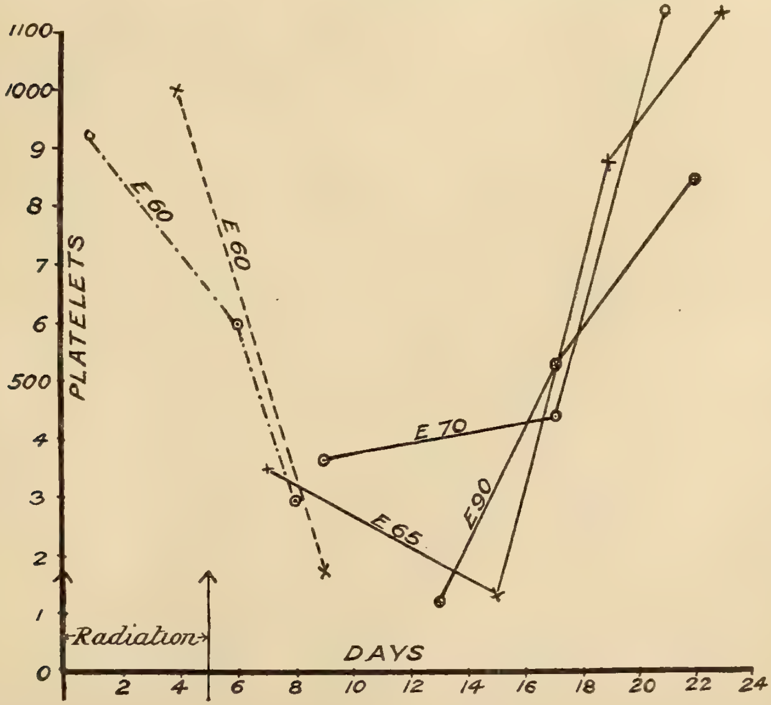

Fig. 5.—Effect of radiation on the number of platelets in five rats. Rapid diminution during and after exposure to radium, followed by rapid recovery.

The changes in the number of the platelets are given in fig. 5, p. 463. A profound thrombopenia develops as a result of the exposure to radium. This persists for a considerable time after the exposure to radium has ceased. It is followed by a rapid spontaneous recovery to the normal number—and sometimes exceeding it—within a week, if the dose of radium has not been too large, as in the present experiment. With larger exposures an anæmia develops and the animals die from intercurrent infections. These findings have an important clinical bearing upon the cases of pernicious anæmia which have occurred among radium workers and which have on a few occasions resulted in death. In all these cases there has been some more or less definite evidence of an infection of the blood stream which has made some hesitate to attribute the condition entirely to exposure to radium. These experiments probably indicate the particular part which micro-organisms play in the manifestation of this type of anæmia; and they indicate the desirability of examining the platelet content in all cases of pernicious anæmia especially of the aplastic varieties.

The Function of Blood Platelets in the Mechanism of Resistance to Bacterial Infection.

The observations recorded in this paper demonstrate a striking relationship between the resistance of an animal to certain bacterial infections and the number of platelets present in the blood. When these latter are diminished below a certain level and kept there for some time, either by withholding the fat soluble vitamin or by exposure to radium, infective conditions develop. When a number of rats are kept in the same cage and on the same vitamin A-free diet, only those animals develop infections in which the platelets have fallen below the critical level. Those rats in which, for reasons given in the paper, the platelets have not been affected to the same extent do not develop these infections. When the deficient vitamin is supplied again the infective conditions clear up as the number of platelets increase, provided of course that these infections have not been allowed to persist for too long a time producing secondary changes such as anæmia, to which the animal eventually succumbs. The infections are as a rule of an avirulent kind. In one case a bacteriological examination was made. Blood cultures from the heart blood showed the presence of two different gram-positive bacilli belonging to the diphtheroid group.

These facts demonstrate that the platelets fulfil an important function in the mechanism of resistance to certain bacterial infections. This conclusion links the observations recorded in this paper to the phenomenon described previously as "defence rupture" or "kataphylaxia" by one of us in conjunction with Dr. W. E. Gye. Cramer and Gye (3) found that the injection of calcium salts, colloidal silicic acid and other colloids and even distilled water produced at the site of injection a diminution in the resistance to infection. They showed subsequently (4) that all these different substances have in common that they produce the same lesion: a damage to the endothelium of the smaller blood-vessels which elicits an agglutination of the platelets within the vessel, and the formation of a white thrombus with a resulting local disturbance in the circulation (see Plate VI, fig. 3, in VI. Scientific Report of Imperial Cancer Research Fund). At the same time lymph and plasma pass out into the surrounding connective tissue where they form a gelatinous clot. When the washed bacteria of gas gangrene or tetanus are injected at the site of this lesion the specific disease (gas gangrene or tetanus as the case may be) is elicited in a very virulent form at this site. The same bacteria when injected into a different site of the same animal do not elicit the disease there but, as in a normal animal, undergo phagocytosis and lysis. At the site of the lesion active phagocytosis is still proceeding, but evidence of lysis has never been observed. This phenomenon of defence rupture is not restricted to the anaërobic bacteria of gas gangrene and tetanus, but holds good also for streptococci, and has recently been shown by Gye and Kettle to be valid also for tubercle bacilli. The lesion responsible for this phenomenon is one which puts the platelets "out of commission," so to speak, *locally* by agglutinating them, and which, by its interference with the circulation, prevents the access of new platelets. This leads to a *local* diminution in the resistance to infection. In the thrombopenia of vitamin A deficiency, or after exposure to radium, there is a *general* absence of platelets, and this leads to a *general* diminution in the resistance to infection.

The literature contains some statements which afford direct evidence that the platelets are concerned in the elaboration of bactericidal substances. The washed platelets and leucocytes do not contain any bactericidal substances when tested against the anthrax bacillus. But when they are mixed with tissue fluids which, by themselves, are inactive, they confer upon this fluid an intense bactericidal power (Gruber and Futaki (5)). It should be noted that these bactericidal substances are not identical with the hæmolytic complement.

More recent work would appear to assign to the platelets a somewhat different function. C. G. Bull (6) has shown in a series of papers that certain bacteria (staphylococci, colon bacilli, meningococci, typhoid bacilli, non-virulent pneumococci and non-virulent influenza bacilli) are rapidly agglutinated when injected into the blood of a normal rabbit or dog. This is followed by a rapid removal of the bacteria from the circulation and by phagocytosis and destruction of the agglutinated bacteria in the capillary systems of the viscera. Those bacteria which are not agglutinated remain in the circulation and produce a progressive septicæmia. Generally speaking, comparing different types of bacteria, the degree of the agglutination of the infecting bacteria in the circulation of the host is a measure of the resistance of the host to the particular types of organism. Bull drew special attention to the fact that with typhoid bacilli, for instance, the mechanism of defence in the living body is very different from that observed *in vitro* by serum or defibrinated blood. In the latter destruction is caused by bacteriolysis, while in the living animal there is the process of agglutination and subsequent phagocytosis in the organs described above. Delrez and Govaerts (7, 8) have followed up these observations, and have shown that this process of agglutination in the living animal is brought about by the platelets. They found that a few minutes after the injection of certain bacteria there is an agglomeration of the platelets and the bacteria. A few minutes later the masses of agglomerated bacteria and platelets can be found in the liver undergoing phagocytosis.

The thrombopenia, which is produced in guinea-pigs by the injection of an antiplatelet serum (the experimental purpura of Ledingham (9)), does not lead to the development of infective conditions, because the animals either die within a few days as the result of the hæmorrhages, or when they recover the thrombopenia rapidly passes off. The thrombopenia alone is, as Bedson (10) has shown, not sufficient to produce hæmorrhages. These are the combined result of the thrombopenia and of a lesion of the vascular endothelium produced by the antiplatelet serum.

Summary.

The absence of the fat soluble vitamin from the diet always leads in the rat to a progressive diminution in the number of blood platelets. This thrombopenia is the only constant lesion which we have found, so far, in every case of vitamin A deficiency, and we regard it as the lesion characteristic of this deficiency, just as the lymphopenia is characteristic of the vitamin B deficiency. A thrombopenia may even be found in rats kept on a vitamin A free diet when they do not yet show any obvious signs of ill-health, and are, to all external appearances, normal animals. When a profound thrombopenia has been established, the addition of the missing vitamin A to the diet is followed by a rapid increase in the number of platelets to the normal number, provided that the animal has not been allowed to suffer too long and too severely from the deficiency.

Exposure to radium produces not only a lymphopenia, but also with sufficiently large doses a thrombopenia. From this the animals recover rapidly, if the application of radium is discontinued, and the dose has not been too large.

If by exposure to radium, or by withholding the fat soluble vitamin, the number of platelets has been reduced below a certain critical level—about 300,000 for the rat—the resistance of the animal to infection is greatly diminished and infective conditions develop spontaneously. These may lead to a secondary anæmia. The infective conditions may clear up again as the number of platelets is made to increase.

The blood platelets fulfil an important function in the mechanism of resistance to bacterial infection. Alterations in the local or general resistance to infection are associated with local or general changes in the distribution of the platelets.

The radium used in these investigations was a loan from the Medical Research Council.

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(8) Govaerts, *ibid.*, vol. 85, p. 745 (1921).
(9) Ledingham, 'The Lancet,' 1914, p. 1673 ; vol. 1, p. 311 (1915).
(10) Bedson, 'Journ. Path. and Bact.,' vol. 25, p. 94 (1922).

The Development of the Calcareous Parts of the Lantern of Aristotle in Echinus Miliaris.

By D. W. Devanesen, M.A.

(Communicated by Prof. E. W. MacBride, F.R.S. Received April 12, 1922.)

[Plates 11–15.]

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1. *Introduction.*

This investigation was suggested to me by Prof. E. W. MacBride, F.R.S., to whom my thanks are also due for kindly placing at my disposal two metamorphosing plutei and a considerable number of young imagos of *Echinus miliaris,* all of them fixed in absolute alcohol.

In the year 1892, Lovén (3) made a few observations on the embryonic elements of the calcareous lantern in *Goniocidaris canaliculata,* a Cidarid with a direct development. He also examined the same structures in two young urchins which, as he himself admits, were of doubtful identity and in a state of bad preservation. In the same year, Théel (7), who was working at the early development of the cake-urchin, *Echinocyamus pusillus,* described certain early stages of the lantern in the young urchins. After these researches of Lovén and Théel, it appears that no attempts have been made to study the development of the lantern ossicles in greater detail and with closer scrutiny.

It must be stated, however, that in the year 1903, in a near relative of *Echinus miliaris,* viz., *Echinus esculentus,* though the origin and homology of the lantern-cœlom had been investigated by Prof. MacBride (4), the calcareous structures it contains remained untouched. One of the obstacles to their study was the fact that non-decalcified specimens could not be sectioned satisfactorily by ordinary methods; and even if they could be sectioned, there was the enormous difficulty of reconstructing, either mentally or otherwise, a very complicated system of ossicles. But, happily, these difficulties were obviated by following a unique idea very kindly suggested to me by Prof. MacBride. He thought that if one could cut away just the top part of the shell, then, after some slight maceration, one could see the lantern ossicles below. By employing this method were obtained several important facts which are recorded in this paper. I here take the opportunity of expressing my deep indebtedness to Prof. MacBride for the many valuable suggestions made and for the frequent advice given throughout the course of this investigation, and for kindly reading the manuscript.

2. *Method.*

The process of double imbedding in celloidin* and paraffin was employed, and the "Jung" sliding microtome was used for sectioning. The latter instrument exactly suited my purpose, viz., making an opening at the aboral surface of the young urchin. Any slight error in orientation can readily be corrected in the course of sectioning by the side-to-side and front-to-rear adjustments of the object-holder. I must remark, however, that it required some skill and judgment to cut away just enough of the aboral part of the shell so as not to damage the lantern below.

When enough of the shell had been sectioned off, the paraffin was dissolved out in xylol, and the celloidin was removed by using a mixture of equal parts of ether and absolute alcohol. The young urchin was then passed through decreasing strengths of alcohol and finally brought into distilled water when it was ready to be treated with "Eau-de-la-barraque." In this liquid was discovered an ideal macerating solution for the young urchin material. While it does not act so vigorously as caustic-potash, for instance, it dissolves in a given time just enough of the tissues to enable one to see through them the contained transparent ossicles. This maceration was watched under a low power of the microscope and it lasted never more than a few seconds. The young urchin was quickly transferred to distilled water and passed through increasing strengths of alcohol, then into xylol, and finally mounted in xylol-balsam in the cavity of an excavated slide. Such whole mounts, when illuminated with a "Nernst" electric lamp and examined with a 2 mm. oil immersion lens, show the ossicles of the lantern very well indeed.

Entire embryonic teeth were obtained by applying gentle pressure, with a mounted-needle under microscopical guidance, to a well-macerated young urchin. The shell breaks away leaving the lantern in the centre. By further manipulations with the mounted-needle the teeth were separated, dried, and mounted in xylol-balsam. An examination of whole embryonic teeth revealed several facts which were hitherto unknown and which threw fresh light on their morphology.

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* The imbedding in celloidin was done according to Gilson's rapid process (Lee's 'Vade Mecum,' 8th edition, p. 104).

3. *The Jaws.*

In describing the development of the lantern ossicles, it is perhaps well to begin with the jaw and proceed upwards. Each jaw, or a pair of "alveoli," is developed from a pair of tri-radiate spicules deposited in an inter-radius of the "echinus-rudiment" of the metamorphosing pluteus; two rays of each rudiment of an alveolus are found embracing a tooth, one on the inner side and the other on the outer side, while the third ray is seen to stretch aborally and towards the rudiment of an "epiphysis" in the contiguous radius (Plate 11, fig. 1, *R.A.*). The next step in the growth of these spicules consists in the branching of the three rays at their ends and in budding new off-shoots over their body; these subsequently join end-to-end. By repetitions of this process, there results a perforated ossicle having more or less the shape of an adult alveolus. In fact, the rudiments of the alveoli, like all other elements of the lantern, with the exception of the teeth, follow the usual method of ossicle growth among echinoderms; but, as compared with the growth of the epiphyses and the rotulæ, for instance, these grow mainly along their vertical axes. This fact enables one to account for the great height of the lantern in urchins generally. This vertical growth of the jaw has caused the ossicles above to be pushed farther and farther away from the oral region. In the very early stages, as may be expected, one finds all the rudiments of the lantern occupying a comparatively low position.

The upward and radially-directed growth of each alveolus results in a junction between it and the rudiment of an epiphysis in the adjoining radius. Each jaw, therefore, bears on the top two epiphyses belonging to different radii.

4. *The Epiphyses.*

One finds their earliest traces as pairs of triradiate spicules situated in each radius of the "echinus-rudiment," far below the rotulæ, and above the level of the jaw-rudiments. Each spicule grows by the usual method, and when a more or less rectangular plate is formed, each epiphysis gives off from its outer corner, in the proximity of a tooth, a process; the two processes belonging to a pair of epiphyses diverge from one another (Plate 14, fig. 5, *O.E.*). One outgrowth meets, over the "foramen magnum" and on the outer side of a tooth, another—I say "another" advisedly, for it is not its fellow—belonging to an epiphysis of a different but adjoining pair (Plate 15, fig. 6, *O.E.*). Both processes together form at this stage a comparatively flat bridge, the arch-form being attained later. It is significant that this bridge was not developed by the palæozoic Echinocystoida, Perischo-echinoida, Cidaroida, Holectypina, and two orders of Centrechinoida. According to Jackson (2, pp. 179 and 183) this is an important character found only in the four families of the Temnopleuridæ, Echinidæ, Strongylocentrotidæ, and Echinometridæ, comprising his new sub-order Camerodonta. Evidently the presence of this bridge for the attachment of the protractor muscles is a sign of advance belonging to a late period in the phylogeny of the sea-urchins.

The two epiphyses connected by their extensions are further in contact below with the same jaw, so that it is customary in text-books to designate them as a pair of epiphyses. But, as far as one could make out from the position of their rudiments, it strikes one that the two epiphyses which lie one on each side of and below a rotula form a pair. This being so, one may say each pair maintains its radial position in the adult, there being no diverging of the epiphyses from a radius. On the contrary, as has been mentioned already, the two alveoli of each jaw, growing aborally like the limbs of a V, each effect a junction with an epiphysis belonging to an adjacent radius. This association, though further strengthened by the formation of a bridge or an arch, is to be regarded as only of secondary importance. The orientation of the ossicles of the lantern in the adult may, therefore, be as follows: compasses, rotulæ, and epiphyses radial; jaws and teeth interradial.

5. *The Rotulæ.*

Each of the five rotulæ is laid down in a radius of the "echinus-rudiment" in the metamorphosing larva, as a tri-radiate spicule, always with two rays directed towards the œsophagus and one ray turned in the opposite direction (Plate 11, figs. 1 and 2, *R.R.*). By the usual process, a broad, fenestrated, and more or less rectangular plate is formed, roofing over the two epiphyses below, which are thus concealed from an aboral view of the lantern at this stage (Plate 14, fig. 5). In this plate, the boundaries of the primordial tri-radiate spicule may still be traced. This stage recalls to one's mind the broad rotulæ found in *Palæodiscus ferox* (6).

6. *The Compasses.*

In the absence of any evidence to the contrary, the "compasses" have been usually regarded as unpaired elements. The bifurcated ends, however, were supposed by Sollas (5) to indicate their paired nature. But their development shows conclusively that, whilst they are duplex structures, the forked ends are not indications of a paired nature.

Each compass can be traced to a pair of tri-radiate spicules placed, not side by side, but in a radial line one behind the other (Plate 13, fig. 13, *I.C.* and *O.C.*). One may, therefore, speak of an outer spicule and an inner spicule with reference to the central axis of the lantern. Nor are these two spicules deposited at the same time as are those of the epiphyses, for instance; the inner first and the outer next is the rule. Further, these two spicules, though in the same line, occupy different positions and levels in relation to the rotula. The outer spicule is situated almost behind the rotula and almost at the same level as the latter, if not below it, whereas the inner spicule is placed just above the rotula. This fact accounts for the bent condition of the compasses in the adult.

These spicules differ somewhat in the manner of their growth, not only from the other members of the lantern, but also between themselves. One of the three rays of the inner spicule is absorbed gradually (Plate 12, fig. 3, *I.C.*) and the remaining two rays stretch end to end, forming a straight rod, more or less, directed radially. The free-branching and anastomosing of the original rays of the rudiment, found in the development of the rotula for example, does not obtain here. The next step in their growth consists in an increase in thickness by the appearance and coalescence of branches at the two ends of the rod; the middle part of the rod appears to grow in thickness all the same, though one does not see as many offshoots there.

The outer spicule has all the three original rays well developed; nevertheless they do not branch. Its growth is confined to its size. The three ends of the spicule appear in a late stage thick and fenestrated, but the body of the spicule is devoid of meshes (Plate 15, fig. 6, *COM.*). The tri-radiate shape is maintained intact and the ray directed centralwards meets the inner piece and forms a suture with it, thus constituting the adult compass. It is at this suture that the elevator muscles of the compasses are attached, some of the fibres to the hinder end of the inner piece and others to the proximal end of the outer. This suture between the two pieces is so complete in the adult echinoids, both extinct and living, that its double nature has been scarcely ever surmised except in one species *Strongylocentrotus dröbachiensis* (Jackson, R. T., p. 179).

The compasses are the only set of ossicles of the lantern which are absent in the "echinus-rudiment" of the larva, but appear after the metamorphosis.

7. *The Teeth.*

At the very outset it may be stated that the tooth is, in the lantern organisation, an element with a unique structure and a peculiar development.

Four stages may be recognised in its construction:—

(1) The formation of a pair of primordial lamellæ;
(2) The deposition of secondary pairs of lamellæ;
(3) The consolidation of all these; and
(4) The attainment of a relatively stable position by the permanently active tooth-germs which will henceforth constitute the root-end.*

The earliest traces of a tooth, so far as I have made out, are an incipient cone, incipient in the sense that, not being completely closed on the outer side, it is a cone in making. It will be seen presently that each cone which comes into being subsequently arises from a pair of lamellæ. One may, therefore, reasonably infer that each of these five cones also arises in the same way from a pair of lamellæ. We shall accordingly call these five cones, the "first cones," and the pair of lamellæ of which each first cone is believed to be composed, the "pair of primordial lamellæ." Each first cone is situated in an inter-radius (Plate 11, fig. 1, *F.C.*), with its apex turned towards the future oral aspect and its base directed towards the future aboral aspect. This cone serves as a starting-point for the upbuilding of the entire tooth; for within the cavity of this cone in the metamorphosing pluteus are found deposited about six pairs of secondary lamellæ. The first cone remains as a distinct structure, even after a good bit of the embryonic tooth has come into being (text-fig. 3, *F.C.*); it is highly probable that finally it enters into union with its successor and forms the tip of the embryonic tooth.

A study of the numerous pairs of lamellæ deposited in succession after the first cone has been formed reveals several facts of no small importance and interest. Each lamella is laid down as a round calcareous particle, which, as I have evidence to show, is probably the product of a single calciferous cell. This particle grows by accretion of lime on one side only in such a way that a small triangular plate is first formed (Plate 11, fig. 1, *L*, and text-fig. 1). At the base of this plate a peripheral growth takes place in one plane, resulting in an imperforate transparent lamella. When it has attained its maximum vertical growth it has got the contour of an isosceles triangle, with its apex directed towards the ventral aspect and its base towards the aboral surface. Even before a particular lamella reaches its full size, fresh lamellæ are deposited at higher levels, one above the other, in quick and regular succession, always on the inner side of the preceding lamella. One can, therefore, read the several stages passed through by a single lamella from the series of lamellæ representing different stages in their growth (text-fig. 2).

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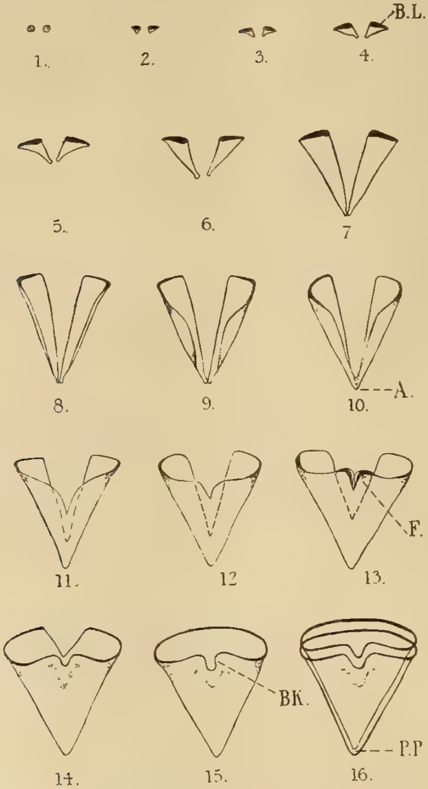
* This fact will be elucidated in a paper on the soft parts of the lantern, now in preparation.



Text-Fig. 1.—Diagrammatic representation of the formation of a cone from a pair of calcareous particles. 1. A pair of calcareous particles. 2, 3, 4, 5, 6, and 7. Progressive stages in the formation of a pair of lamellæ; *B.L.*, Basal end of a lamella where growth takes place by accretion. 8. The pair of lamellæ has attained its maximum vertical growth, further increase taking place only at the lateral edges; the two lamellæ are undergoing slight bending due probably to the pressure of lamellæ subsequently deposited. 9 and 10. The inner lateral edges of the two lamellæ have commenced to grow; *A.*, The first spot where the latter come into contact and fuse. 11 and 12. The inner lateral edges have approximated and are gradually fusing; the outer lateral edges have also begun to join; it will be noticed that both the commencement and the completion of the fusion of these latter edges are later than those of the inner; this stage in the formation of a cone may be called the "incipient cone" stage. 13. The flange *F.*, at the top corner of each inner lateral edge, has appeared. 14. The same flanges have joined, forming the characteristic beak. 15. A complete cone; *BK.*, Beak. 16. Two cones showing their relation to each other; *P.P.*, the spot where the fusion between the apices of the two cones takes place, this being the foundation for the formation of an axial rod—the "pars petrosa."

The relative position of the two fellow lamellæ of a pair deserves notice. They are deposited in close proximity to each other, but do not touch, and also at different levels so that they alternate. As growth proceeds, however, the two fellow lamellæ are found at the same level, inclined, at first, towards each other at an angle which varies with their growth till, finally, as the result probably of pressure from succeeding lamellæ, their apices are brought into contact and fuse together.

At this stage of our investigation it is, perhaps, well to take into account certain fundamental facts revealed to us:—

(1) We see that a lamella does not pass through a tri-radiate spicule condition, unlike, in this respect, the other echinoderm ossicles which do, as far as is known at present.

(2) The growth of a lamella is unusual, being different from the normal method of ossicle-formation which obtains among the other component elements of the lantern.

(3) There is a maximum limit to the vertical growth of a lamella, further growth being along the two equal edges of each lamella.

(4) From the fact that numerous pairs of lamellæ go to build the adult tooth, two conclusions can be drawn, viz., (*a*) that the urchin-tooth is a paired structure, and (*b*) that a pair of lamellæ is the unit of the same. This admission of the morphological value of a pair of lamellæ as a unit is not, as will be seen later, prejudicial to regarding a cone formed by a pair of lamellæ as an integral structure.

The process of formation of the tooth from these paired lamellæ is not by any means simple.* When two lamellæ of a pair have attained their maximum vertical growth, by the pressure probably of the succeeding lamellæ above, they become bent and concave to an equal extent on their inner surfaces, their outer surfaces becoming correspondingly convex. During this bending the two lamellæ appear to grow along their lateral edges till those of one lamella face each to each their two fellows on the other lamella (text-fig. 1). This growth is maintained till the confronting edges meet and fuse, forming a characteristic cone.†

While the fusion of the inner edges is taking place, the rudiments of the carina are found to arise. An inwardly directed flange appears at the top corner of each inner edge (text-fig. 1). At about this time each cone undergoes a flattening from the inner to the outer side. Partly as the result of this flattening and partly owing to their growth, the above-mentioned flanges project well towards the œsophageal side. They ultimately come into contact and fuse forming a beak-shaped process somewhat like the beak of an ounce-glass. It will be seen that in the formation of this beak both lamellæ take an equal share. When several cones fit into one another, their beaks likewise fit together, thus giving rise to a crest which is the precursor of the carina of the adult tooth. The lower part of the tooth in the imago appears to be devoid of this crest; only the cones arising later develop the beaks in question.

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* Lovén's (3, p. 9) description of the formation of a tooth, in *Goniocidaris*, from a single row of lamellæ is extraordinarily simple. He says as follows: "The lamels, the general form of which corresponds with that of the tooth, are laid one upon another regularly from the top downwards." Spencer (1904, p. 36), likewise, figures a simple arrangement of lamellæ in *Palæodiscus ferox*.

† Théel (7) describes also a cone-in-cone arrangement in *Echinocyamus pusillus*.

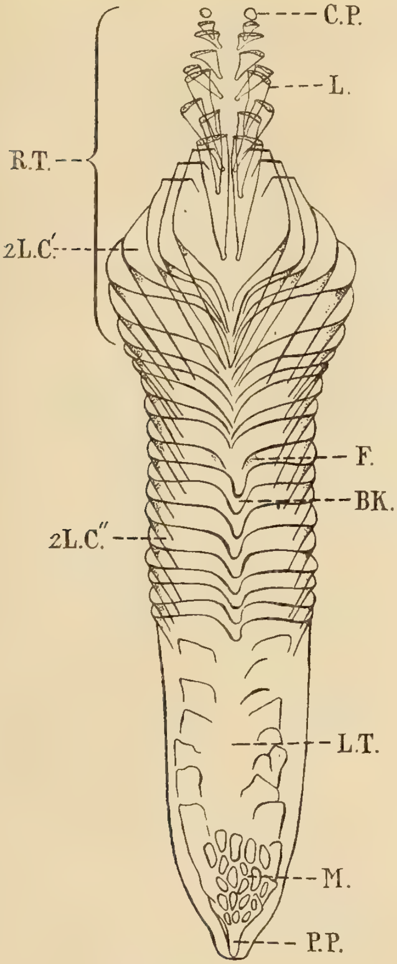
The fitting into one another of the cones takes place in such a manner that the brim of an upper cone always projects a small distance from above the brim of the lower cone into which it is enclosed. This fact has a necessary bearing, first, on the growth of a carina on the inner side and, second, on the formation probably of the middle furrow on the outer side of each tooth (Plate 15, fig. 6, *CA.* and text-fig. 1).

In the aboral end of the embryonic tooth—the root-end in the adult—one can see all the intermediate stages between a pair of calcareous particles, the simplest condition of a pair of lamellæ, and a transparent beaked cone, the most advanced stage of the same (text-fig. 2). As has been already mentioned, the first point of contact and fusion between every two lamellæ of a pair is at their apices. The fused apices of one pair unites with those of its predecessor and successor even before the pair itself reaches the stage of a cone. Thus a central rod results to which are attached all the cones and the younger pairs of lamellæ. Presumably this rod is the "pars petrosa" of Giesbrecht (1, p. 90). Curiously enough, the first cone does not appear to participate in the formation of this stony part (text-fig. 3).

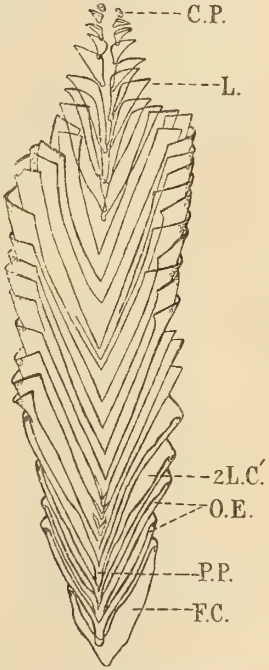
It has not been possible to follow closely the next phase in the building of the tooth. The coalescence of the cones appears to proceed centripetally from the "pars petrosa" outwards. The outlines of the cones gradually disappear owing to the fusion of their walls.

The median furrow of the adult tooth probably marks the place where the suture of the two outer edges of a pair of lamellæ is formed. Of this line of junction a greater portion is bound to disappear in the process of coalescence owing to its internal position in the cavity of its predecessor as explained already; only the small exposed parts of this suture in the several cones probably persist and contribute to the formation of the median furrow.

On account of the relative position between every two cones, the beaks are, on the inner side, the only exposed parts of the line of junction between the two inner edges of the pairs of lamellæ. It must be observed that no indication of a suture, however, is to be found on each beak. These beaks, as has been already described, are the precursors of the carina which is for this reason a composite paired structure. Such is the construction of a tooth so far as one could make out from observations of hard structures.



TEXT-FIG. 2.



TEXT-FIG. 3.

TEXT-FIG. 2.—Reconstruction of a fairly advanced tooth as viewed from the inner side. *R.T.*, Root-end of the tooth where the lamellæ can be seen in all their progressive stages; *C.P.*, Calcareous particle, the first beginning of a lamella; *F.*, Flange; *L.*, Lamellæ: 2*L.C.′*, Two lamellæ in the process of cone-formation; 2*L.C.″*, A fully formed cone; *BK.*, The beak of a cone; *L.T.*, Lower part of the tooth where the walls of the cones have fused; *M.*, Mesh-work formed by the coalescence of lamellæ; *P.P.*, "Pars petrosa," or the stony part.

TEXT-FIG. 3.—Actual drawing of a tooth less advanced than the one shown in text-fig. 2, viewed from the outer side; the first cone is still intact; it will be noticed that the cones are yet imperfect on the outer side, fusion between the edges thereof being considerably delayed; *F.C.*, First cone; *P.P.*, the "Pars petrosa," or the stony part resulting from the union of the apices of the serially fitting cones; 2*L.C.′*, Two lamellæ in the process of cone-formation; *L.*, Lamellæ; *C.P.*, Calcareous particle; *O.E.*, Outer edges not yet approximated.

8. *Discussion.*

In the mouth-frame of recent Asteroidea as well as in most of the fossil star-fishes are seen five pairs of generally triangular ossicles situated in the inter-radial angles bordering the mouth (Spencer, 1913, p. 26). Originally identified by Ludwig as the first pair of modified adambulacrals, they are now known as mouth-angle plates. By their position both in the embryo and in the adult and by their relation to the mouth, the five pairs of "alveoli" of the lantern of Aristotle recall the five pairs of mouth-angle plates in star-fishes.

In the section on "epiphyses," we saw how the two epiphyses each situated on an alveolus belonging to adjacent jaws constituted a pair, and not the two epiphyses which formed an arch. As the radial canal of the water-vascular system runs between the two epiphyses in question,* there is no serious obstacle to regarding this pair as corresponding to a pair of ambulacral ossicles.

The interpretation of the rotulæ offers undoubtedly great difficulties. These are not only odd elements as conclusively shown by their origin but also the sole unpaired elements in the lantern of Aristotle. The only odd ossicle which gets into touch with the mouth-frame in extinct star-fishes is the odontophor. In an account of the family Urasterellidæ, Spencer (1913, p. 135) observes as follows :—"In a private letter to me . . . . Hudson asks me to note (in the mouth-frame of the American species *Urasterella pulchella*)† 'paired muscle remains . . . . just within the large inter-radial (odontophor).' He goes on to state that there must have been muscles to draw in the mouth-angle plates, which in each inter-radius acted as an outer jaw. The origin of these adductors may have been on the inner surface of the odontophors rather than on the first ambulacralia." This indicates that in certain asteroids the odontophor may get into close relationship with the mouth-angle plates. It must be noted, however, that in the lantern of Aristotle the rotula has muscular connection with the epiphyses and not with the jaws.

In certain other fossil star-fishes again, the odontophor has been shown to occupy an internal position (Spencer, 1917, p. 180). I quote these two features of the odontophor in the extinct asteroidea in order to show how this odd ossicle behaves in certain cases, it being far from my purpose to suggest thereby any asteroid descent for the echinoids.

The great impediment to regarding the rotulæ as modified odontophors is the fact that, while the latter are inter-radial, the former are radial. Can it be that, after occupying an internal position in the ancestral urchin, the odontophors underwent a sinistral or dextral rotation which brought them to a radial position? MacBride* with reference to the inter-pyramidal muscles says as follows: "These on contraction, approximate the pair of jaws into which they are inserted, and it will easily be seen that by the successive contraction of the five comminator muscles a rotating movement of the teeth would be produced which would cause them to exert an action something like that of an auger." Can this action of the lantern, coupled with the fact that the rotulæ alone of all the ossicles of the lantern are free from muscular attachment with the shell, be supposed to have brought about this displacement?

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* This fact will be elucidated in a paper now in preparation.

† This parenthesis is mine.

From its double origin and radial position each compass may be regarded as corresponding to a pair of ambulacral ossicles. It is generally known that in certain extinct star-fishes, the paired ambulacral plates alternated with each other. If the same condition had prevailed among the ancestors of urchins, one might conceive of a displacement consisting of one member of a pair being pushed in front of his fellow.

In instituting a comparison between a tooth and other ossicles of the lantern or those of the mouth-frame of star-fishes, one should take a pair of primordial lamellæ to represent a tooth on the one hand and paired ossicles on the other. For I hold an urchin-tooth is not, in the strict sense of the term, an ossicle; it is an aggregate of paired ossicles if the lamellæ can be so called. Whether the lamellæ themselves are ossicles is open to doubt; as we saw in their development, they neither pass through a tri-radiate spicule stage nor do they grow into fenestrated plates as ossicles of echinoderms do in general by the branching and anastomosing of calcareous offshoots. On the contrary a tooth-lamella grows by accretion confined in the early stages to one particular side, viz., the base of the minute triangular plate. For purposes of comparison, therefore, a pair of tooth-lamellæ may be, with the above-mentioned reservations, taken to represent a pair of ossicles corresponding to a pair of "alveoli" or "epiphyses" rudiments. This being so, a whole tooth does not stand in the same relation to its rudiments as the other component parts of the lantern do; the latter are, *par excellence*, echinoderm ossicles whereas the former is an aggregate of paired structures which are not undoubted ossicles. An epiphysis for instance, being the direct outcome of a tri-radiate spicule, is a unit in itself; but a tooth is an aggregate, being the product of several paired units, the lamellæ. A tooth is essentially a double structure like a pair of "alveoli" or "epiphyses," the only indication of this in the adult tooth being the median furrow which runs longitudinally along its outer side.

The homologues of the urchin-tooth are to be looked for among the bristles carried by the mouth-angle plates in star-fishes. If two such bristles get flattened, assume a cone-shape, and are pushed between the two mouth-angle plates thus becoming partly internal, we have the rudiment of an urchin-tooth.

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* See "Echinodermata," 'Cam. Nat. Hist.,' p. 526.

There must have been several physiological forces at work in the evolution of the urchin-tooth. First of all, the liability to wear and tear of an organ used for browsing purposes could have induced the permanent retention of the activity of the embryonic tooth-germ. The ancestor of the sea-urchin, whoever that might have been, let us suppose, started with the five first cones; these may well have served the primitive animal as organs of mastication. If the ancestral animal browsed on things like the brown fronds of *Laminaria* or bored into rocks*—certain sea-urchins are known to do both—the conical teeth would be liable to suffer decay. Under such circumstances, the power of replacing worn-out teeth would have been of immense advantage, and hence the permanent activity in the adult of the five embryonic tooth-germs was probably the primary factor in the evolution of the urchin-tooth.

But how can one account for the coming into being of a stout rod as the result of fusion of several pairs of lamellæ? What could have induced the deposition of numerous pairs of lamellæ, one above the other, in the imago urchin, while even as yet the mouth is not formed? Efficiency is the first answer that suggests itself. A short conical tooth, formed by a pair of delicate lamellæ and renewed frequently, even at its best must have been but a weak instrument to the ancestral urchin. If the tooth-germs laid down precociously pairs of lamellæ, and if by the fusion of these a stout rod resulted, that meant efficiency in function and advance in structure. In this way, one may account for the formation of a stout rod-shaped tooth through the fusion of several cones.

In this connection, perhaps, it will be well to consider what reaction the evolution of the tooth may have had on the jaws. Each tooth in the adult is closely and immovably attached to a jaw. This being so, the jaw is bound to respond to any adaptations of the tooth. In the "echinus-rudiment," the vertical height of the lantern is at its minimum.† Now, if the tooth increases in length in accordance with the causes indicated, it is extremely likely that there will be a corresponding response on the part of the jaw to adjust itself to the growing tooth. That the tooth may well have been the leading factor in the evolution of a high lantern is perhaps seen in the lantern of the metamorphosing pluteus (Plate 11, fig. 1, *L*). Here we find that, even while the two jaw-rudiments are in the tri-radiate spicule stage, the tooth is in a relatively higher state of development with the first cone and nine pairs of secondary lamellæ; and it extends aborally beyond the limits of the jaw-rudiments. It is perhaps admissible to infer from this that, with the growing length of the tooth, the jaw-rudiments kept pace *pari passu*, and the result was a lantern of great height.

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* *Vide* "The Locomotor Function of the Lantern in Echinus," by Prof. Gemmill, 'Roy. Soc. Proc.,' vol. 85, p. 101 (1912).

† The height of the lantern in *Palæodiscus ferox* is small when compared with that of living urchins (Spencer, 1904).

"Summarising the foregoing conclusions, I regard the lantern of Aristotle as homoplastic with the buccal armature of star-fishes; the pyramids are the modified first adambulacral plates; the epiphyses have arisen from the first ambulacral plates of the Echinoid series; and the teeth represent the odontophore, which has acquired a persistent root; the radius and rotula remain problematical." So wrote Prof. Sollas in the year 1899 in his paper on "Silurian Echinoidea and Ophiuroidea" (5). I leave the reader to compare this statement with what has been indicated on the homologies of the lantern of Aristotle in this paper. It will be seen that the idea of the homology of the urchin-tooth advanced by me is entirely new, and based on indisputable embryological facts.

9. *A Comparison between Giesbrecht's* (1)* *Account of the Development of the Urchin-tooth as revealed by its Root-end in the Adult and the Construction of the Same as seen in the Young Urchin.*

Attempts to probe into the peculiar construction of an urchin-tooth were made even as early as the year 1841. Beginning with Valentin,† Meyer, Waldeyer, Leuckart, Giesbrecht, Lovén, Théel and MacBride successively each gave his attention to this organ. With the exception of Lovén, Théel and MacBride, who studied it in young urchins, these authors employed in their researches the root-end of the adult urchin-tooth. The credit of our knowledge that the urchin-tooth is built up of lamellæ is largely due to these workers. Nevertheless, our ideas as to the manner of the origin of these lamellæ, their growth, arrangement and significance have hitherto been rather imperfect. The first attempt, I may say, to interpret the tooth-structure in terms of biogenetic laws has been made in this paper (see Discussion).

A very interesting study was made by comparing my own observations, based on the embryonic tooth, with those obtained by the examination of the root-end of an adult tooth. From the nature of the case one should expect the process in the adult to be similar to that in the young. Hence a comparison between the two is inevitable, nay, compulsory. I am bound to confess that my own researches do not confirm in their entirety the conclusions drawn by Giesbrecht, the last and most important of the investigators of the root-end of the adult urchin-tooth.

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* The greater part of this important paper was translated to me by Prof. MacBride.

† "Anatomie du genre Echinus," 1841, by G. Valentin, the first monograph in 'Monogr. d'Echinodermes viv. et foss.,' published by L. Agassiz.

According to his observations there are two sets of units for the urchin-tooth, one for the wings or the lateral parts and the other for the carina. The units of the former are flat structures called "Scales" (Schuppen), which are not homogeneous but are made of two lamellæ separated by a narrow interspace. It appears to me that, in this latter inference, Giesbrecht has been misled by an artifact, and, likewise also in his other, mentioned in the context, that his second set of units, "the prisms," have an axis-cylinder. Each scale is undoubtedly a homogeneous structure of integral value. For this reason, and also because it is more appropriate, I have, in the description of these plates, retained the use of the term "lamella," and dropped out the word "scale." His account of the manner of growth of each "scale" is not in perfect accord with mine. He says: "Meanwhile, quite like the shell of a mussel, lime is deposited in layers round one 'initial point' (the 'calcareous particle' in my description),* though not uniformly in a circle but on one side only, so that the initial point always remains at one edge of the plate like the umbo of a mussel shell." Though this description suggests a peripheral growth, it differs in two respects: (1) the growth in the pre-cone-formation period is by accretion in a straight line along the base of the triangular plate (text-fig. 1); and (2) the concentric rows of stripes he mentions in the context are non-existent in the lamella. Further, Giesbrecht describes the lamellæ of one row as alternating with those of the other, and has entirely missed out the formation of the cones.

To me, it would appear, that his second set of units—what he calls "prisms," but, in reality, long needle-shaped structures—is of the nature of secondary calcification. In the young imago, the carina is found to arise solely by the fusion of inwardly directed flanges (text-fig. 1) of the incipient cones, as has been already described in the section on teeth. Giesbrecht himself speaks of a certain part of the lamella taking part in the formation of the carina. I cannot, therefore, agree to his giving the "prisms" a morphological value equal to that of the lamellæ.

On account of the nature of the methods he employed, such as dissecting the root-end with a mounted-needle and making ground-sections (Dünnschliffen) of the same, I do not know how far one can rely on these doubtful details of his observations. He boiled the root-end with caustic-potash, a treatment which undoubtedly would have interfered with the structure of such delicate things as the lamellæ. The wonder is that Giesbrecht accomplished so much with the methods at his disposal in those days.

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* The parenthesis is mine.

10. *Summary.*

(1) All the calcareous elements of the lantern of Aristotle, with the exception of the teeth, are deposited as tri-radiate spicules; in this, as well as in their further growth, they resemble the ossicles of echinoderms in general.

(2) The two "epiphyses," one on each side of and below a rotula, are to be regarded as constituting a pair.

(3) A "compass" arises from two rudimentary spicules. It is the only element of the lantern absent in the "echinus-rudiment."

(4) A tooth is a paired structure in consequence of its composition of a double row of lamellæ. A pair of lamellæ is its ultimate unit, although it is not inconceivable that originally a pair of lamellæ itself, after assuming a cone-shape, could have functioned as an integral structure, a primitive kind of tooth, in the ancestral urchin. There is a remarkable stage in the consolidation of these lamellæ, viz., the cone-in-cone arrangement. The carina is formed by the beaks of the serially-fitting cones.

(5) Without committing one's self to the view of a direct descent of the sea-urchins from the star-fishes, one may institute a brief comparison between the ossicles of the lantern and those of the mouth-frame of a star-fish: a pair of "alveoli" corresponds to a pair of mouth-angle plates; a pair of "epiphyses," as understood in this paper, to the first pair of ambulacrals; a pair of compasses to the second pair of ambulacrals; the rotula may, tentatively, be regarded as a displaced odontophor; the pair of primordial lamellæ, the forerunner of the urchin-tooth, may be compared to a pair of bristles attached to a pair of mouth-angle plates.

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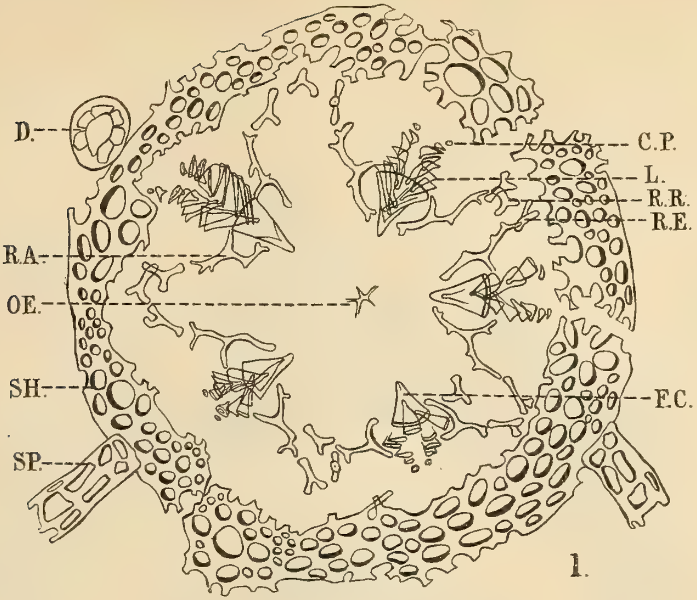
EXPLANATION OF PLATES.

List of Abbreviations used.

A.	Alveolus.
B.	Boss of a spine.
BK.	Beak of a cone.
B.L.	Basal ends of lamellæ.
CA.	Carina of a tooth.
C.P.	Calcareous particle—the starting point of a lamella.
COM.	Compass.
D.	Calcareous disc of a tube-foot sucker.
E.	Epiphysis.
EL.M.	Elevator muscles of a compass.
F.	Flange of a lamella.
F.C.	First cone.
I.C.	Inner rudiment of a compass.
IN.M.	Inter-pyramidal muscles.
L.	Lamellæ of a tooth.
2*L.C.'*	A pair of lamellæ in the process of cone-formation.
2 *L.C."*	A fully formed cone.
O.C.	Outer rudiment of a compass.
OE.	Œsophagus.
O.E.	Offshoot of an epiphysis.
O.T.	Oral end of a tooth.
P.E.	A pair of epiphyses.
R.	Rotula.
R.A.	Rudiment of an alveolus.
R.E.	Rudiment of an epiphysis.
R.T.	Root-end of a tooth.
R.R.	Rudiment of a rotula.
SH.	Shell.
SP.	Spine.
S.C.	Suture between the two rudiments of a compass.
S.E.	Suture between the processes of two epiphyses.
T.	Tooth.

As the six figures form a progressive series, all of them should be consulted with regard to any particular ossicle of the lantern of Aristotle.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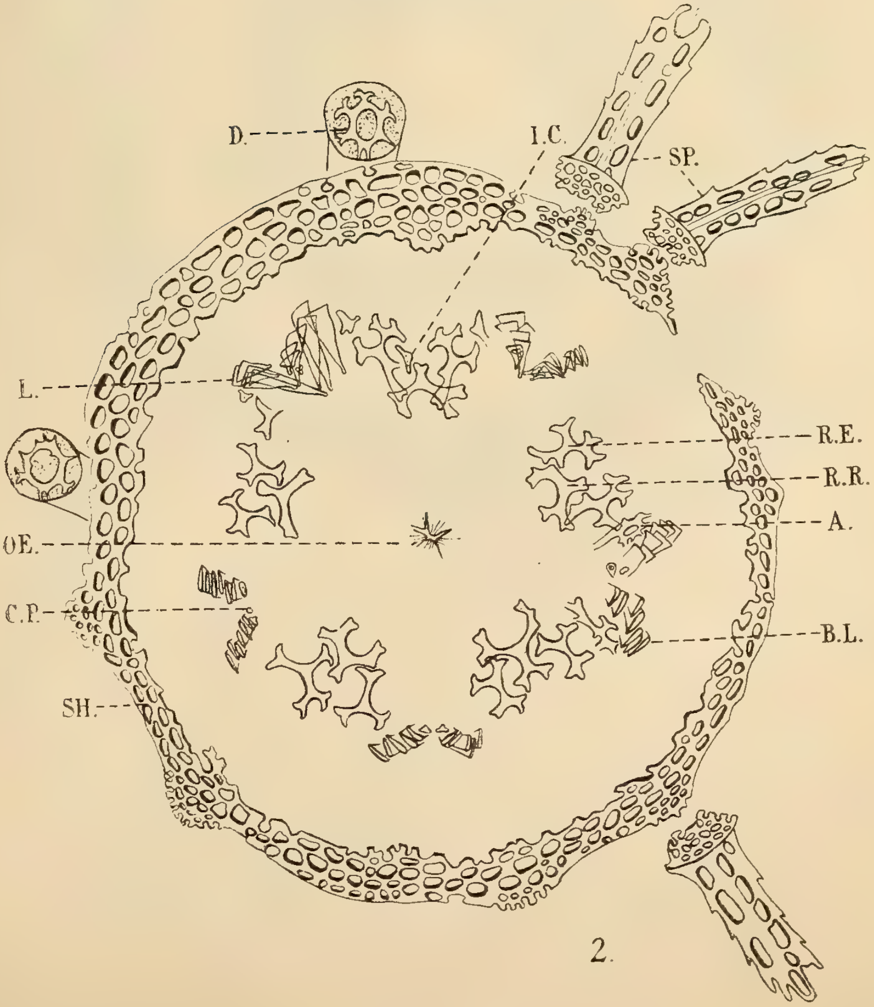
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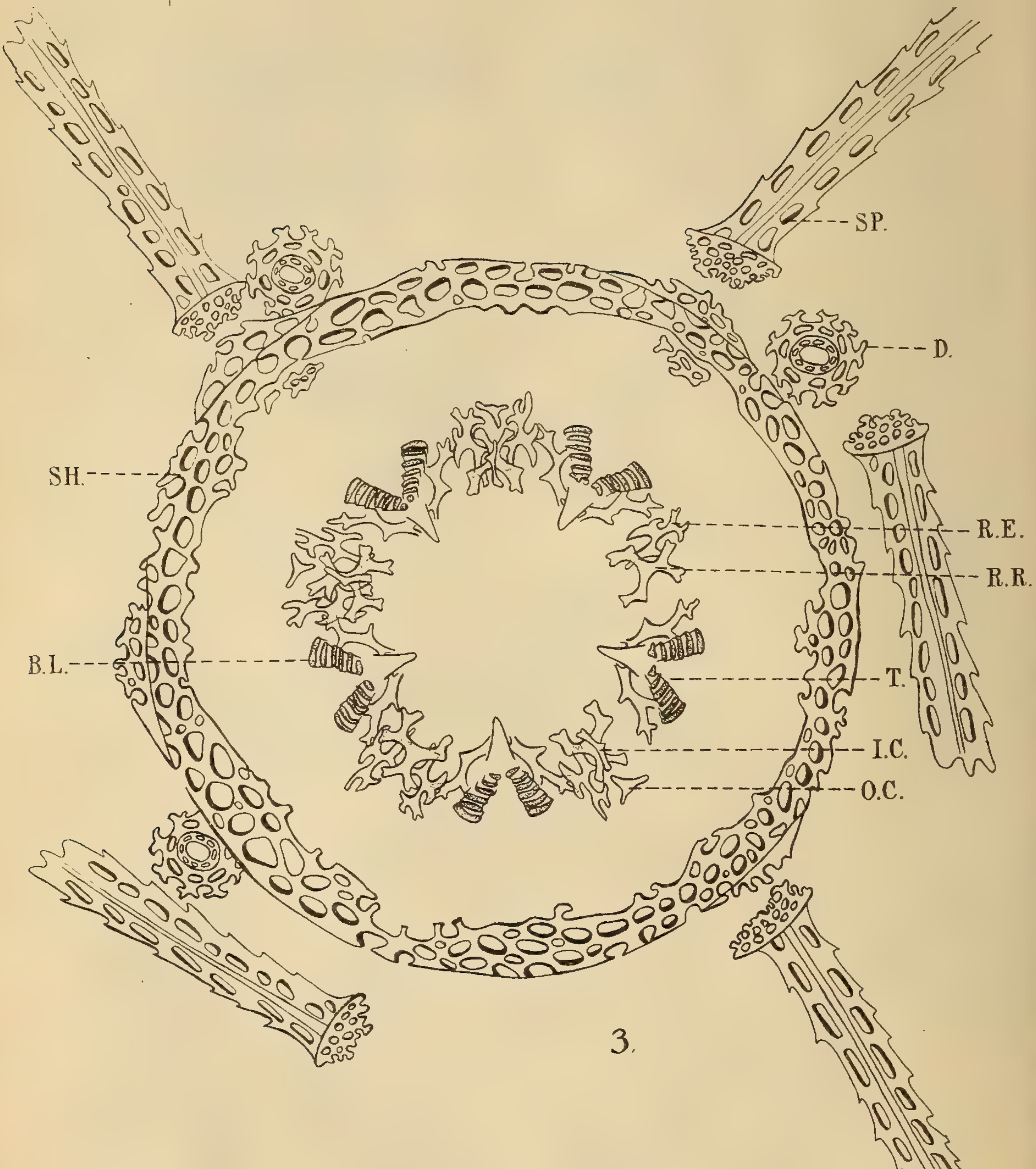
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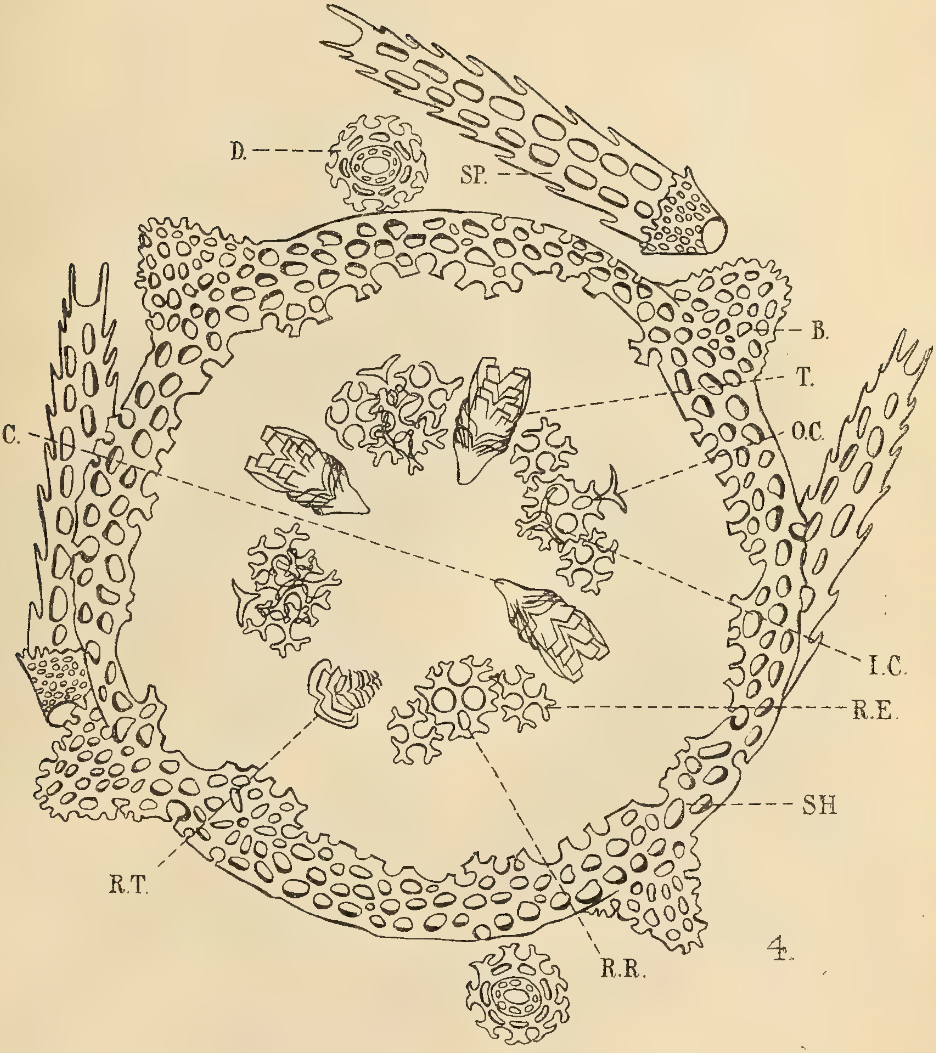
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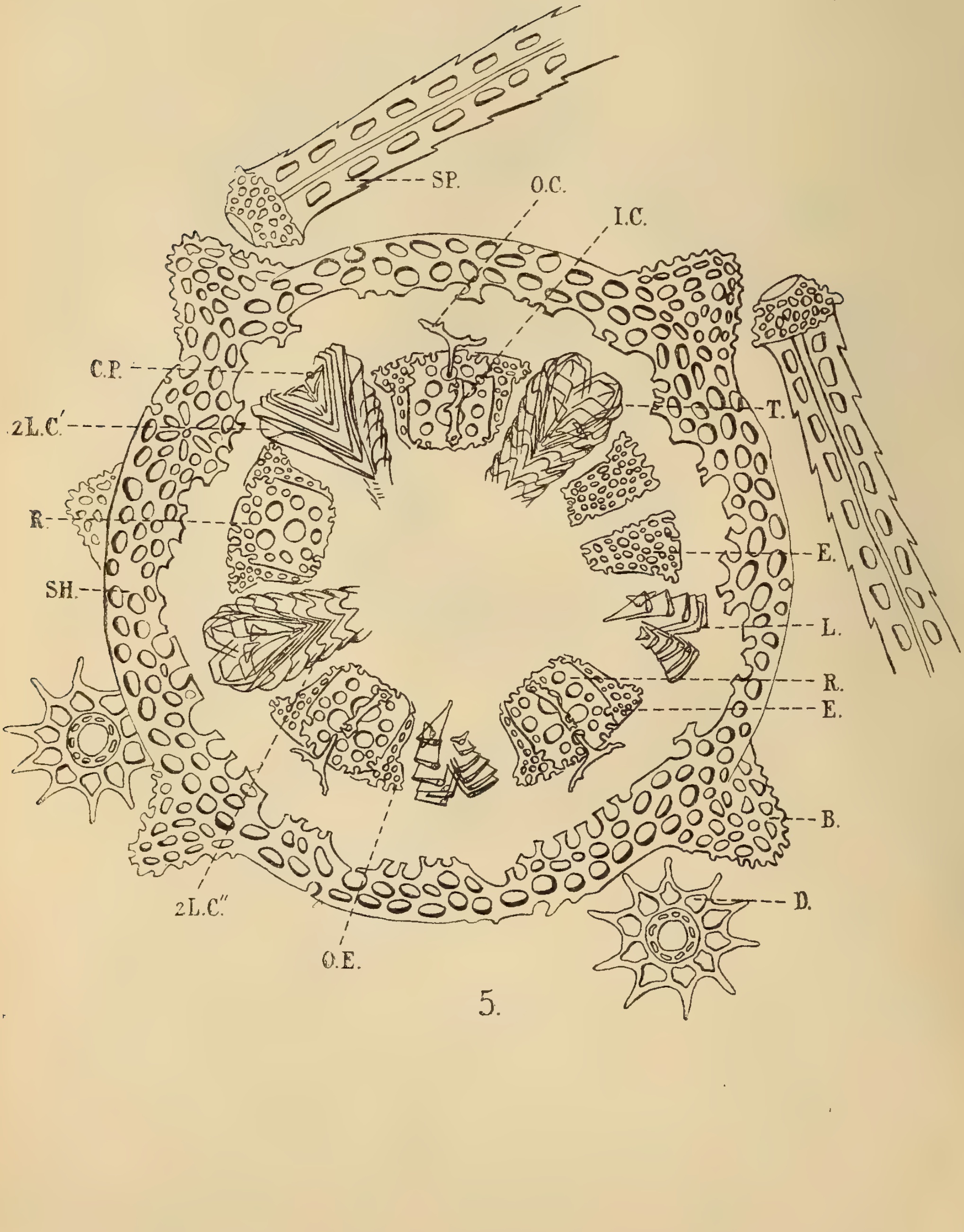
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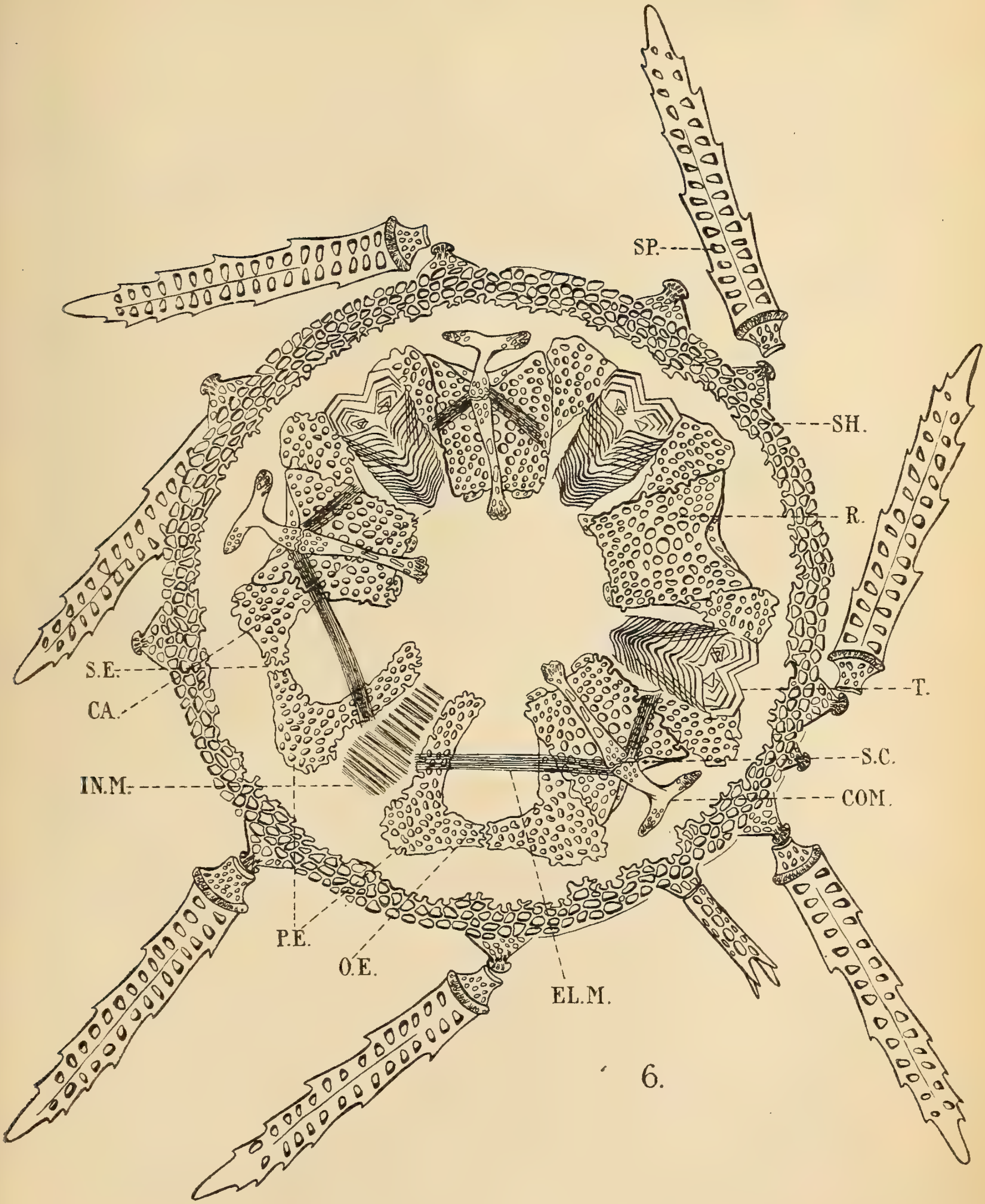
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EL.M.

6.

DESCRIPTION OF PLATES.

Plate 11.

Fig. 1.—"Echinus rudiment" of a metamorphosing pluteus; macerated after all the extra-larval parts have been removed by sectioning; the lantern elements are seen from the future aboral surface; they are all, with the exception of the teeth, in the stage of tri-radiate spicules; no trace of compasses.

Fig. 2.—Aboral view of the lantern elements in a young imago, probably just metamorphosed; the top part of the shell has been removed and the specimen macerated; the inner rudiment of a compass visible in one radius; branching of the rays has commenced in all the spicules.

Plate 12.

Fig. 3.—Aboral view of the same in an imago-urchin slightly more advanced; well-marked rudiments of the compasses are laid down in all the radii; epiphyses considerably branched; the teeth are rather diagrammatically represented.

Plate 13.

Fig. 4.—Aboral view of the same in imago still more advanced; the first branches of each rotular rudiment have joined end to end and fresh off-shoots have been put forth; one ray of the inner rudiment of each compass is in process of resorption and another ray is being lengthened to meet the outer rudiment; beaks are not formed by the earlier-produced cones; the root-end of a tooth shows the characteristic bend of the same seen in the adult; a compass has been omitted in one radius; it will be noticed the specimen is tetramerous in symmetry.

Plate 14.

Fig. 5.—Aboral view of the same in imago more advanced than the one shown in fig. 4; the rotulæ and the epiphyses have grown into fenestrated plates; the outline of the original spicule may yet be traced in each rotula; the two rudiments of each compass are still separate; the epiphyses are putting forth processes; the alveoli are not visible; the compasses in two radii and a rotula in one radius have been omitted.

Plate 15.

Fig. 6.—Aboral view of the same in a fairly grown young urchin; the rotulæ are beginning to sink between the two epiphyses below; the off-shoots of the latter have nearly met across the "foramen magnum"; the two pieces of each compass have met and formed a suture; a carina in each tooth can now be distinctly seen; the jaws are not visible; a rotula and a compass have together been omitted in one radius in order that a pair of epiphyses may be brought into view; likewise, the teeth have been left out in two radii to show the bridge formed by the processes of two epiphyses; the compass alone has been omitted in one radius to bring a rotula into full view.

Origin and Destiny of Cholesterol in the Animal Organism.
Part XIII—*On the Autolysis of Liver and Spleen.*

By JOHN ADDYMAN GARDNER and FRANCIS WILLIAM FOX (Beit Memorial Fellow).

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(Report to the Medical Research Council. From the Biochemical Laboratory, St. George's Hospital, and the Physiological Laboratory, South Kensington, London University.)

In Part XII of this series (1921) the comparison was made of the intake and output of cholesterol in normal adults on a known diet and over periods of six days, and an average daily loss of 0·3 grm. of cholesterol was noted. The conclusion drawn was that there must be some organ in the body capable of synthesising cholesterol. A similar view has been put forward by Grigaut (1913), who expressed the opinion that this synthesis is the function of the suprarenal glands. It seemed likely that the study of the autolysis of various tissues under aseptic conditions might throw some light on this question.

A number of observations bearing on this subject are described in the literature, some undertaken with the object of finding evidence of the presence of enzymes capable of hydrolysing cholesterol esters, others with the object of ascertaining whether destruction or synthesis of cholesterol took place on autolysis. The results are very conflicting.

In 1896, Carbonne, and again Waldvogel, in 1906, claimed to have obtained a large increase in cholesterol when lecithin was autolysed with sterile liver juice. Craven Moore (1907), however, was unable to confirm this, and described one experiment in which 600 grm. of human liver, containing 0·037 per cent. of cholesterol were autolysed aseptically for 42 days at 37° C., and at the end were found to contain 0·038 per cent. Corper (1912) also failed to find any marked change in the cholesterol of dog spleen on short autolysis.

Kondo (1910) attempted to decide the question of the presence of enzymes capable of hydrolysing cholesterol esters in blood and tissues by the determination of the acetyl values of the ether extracts, but his conclusions were vitiated by the presence in addition to cholesterol of other acetylisable substances.

In 1912, Schültze repeated these experiments, using the digitonin method for estimation of cholesterol and its esters. He found that in autolysis of human blood and horse blood no hydrolysis of cholesterol esters took place.

Rabbit's liver gave negative results, but in the case of horse liver a marked hydrolysis took place. In experiments with mixtures of blood and liver-juice, both in the case of horse and cow, a more or less complete hydrolysis took place, but not with serum and liver-juice. Schültze made no comment on the constancy or otherwise of the total cholesterol on autolysis, but as far as we can ascertain from his protocols, any variation between the values of the fresh and autolysed tissues were within the probable errors of experiment.

Cytronberg (1912) continued the work of Schültze and showed that the cholesterase is contained in the blood cells and not in the plasma. Howard Mueller (1916) however was unable to confirm Cytronberg's work.

In 1920, T. E. Abelous and L. C. Soula found that spleen pulp on autolysis at 37° showed a marked increase in total cholesterol. Some of their results are given in the following table:—

Percentage of Cholesterol in Spleen Pulp.

Animal.	Fresh.	After 24 hours autolysis.	48 hours.	6 days.	10 days.
Dog	0·231	0·930	—	—	—
Calf	0·500	0·580	0·680	0·399	0·150
,,	0·350	0·450	0·150	—	—
,,	0·078	0·459	—	—	—
Cow	0·317	0·570	0·415	—	—

Initially, it will be noticed, they found a rise followed on prolonged autolysis by a fall. They also found that these effects increased with rise of temperature up to 45° C.

In one experiment the addition of a minute amount of cholalic acid produced a still more marked synthesis of cholesterol.

Animal.	Initial content.	48 hours.	48 hours (with 0·05 grm. cholalic acid added).
Calf	0·500	0·68	1·11

They also investigated other organs and concluded generally that nervous tissue and liver also have the power of synthesising cholesterol, but in less degree than spleen. Other tissues on autolysis show a destruction of cholesterol.

The authors give no details of their methods of extraction and analysis, but obviously their results, if correct, are of very great importance.

We determined, therefore, to study the autolysis of various organs, and in this paper give an account of our experiments on liver and spleen.

Method.

The whole fresh organ, freed as completely as possible from adhering tissue and fat, was finely minced in a mincing machine, pulped in a mortar, and divided into the required number of approximately equal portions, which were then accurately weighed. One portion was analysed at once and the others mixed, in some cases, with sufficient toluene, and in other cases with a 2 per cent. solution of sodium fluoride to prevent bacterial growth. They were then placed in sterile flasks in the incubator at 37° C. and allowed to autolyse for definite periods. The autolysed tissue was not examined bacteriologically, but the smell indicated that no appreciable putrefaction had taken place.

Extraction of Fat.

This is the most important stage of the process, and one in which, in our experience, error is most likely to occur. The pulped tissue—fresh or autolysed—was mixed with excess of alcohol and the fluid portion then drained off. The solid matter was placed in a paper thimble and extracted with alcohol for several days, the material being taken out at intervals and re-ground. Finally the extraction was completed by means of ether.

The alcoholic fluids were evaporated and the residue thoroughly extracted with ether. The ethereal extract was added to the above and made to known volume. An aliquot portion was evaporated to obtain the total ether extract, and, when desired, to estimate the free cholesterol.

The extraction was carried out in a flask or matrass with a very long wide neck. The paper thimble was placed in an ordinary Soxhlet tube with syphon, suspended by a platinum wire in the neck of the flask. Above this was suspended a closely wound glass spiral tube through which cold water circulated, and which served as a very efficient condenser. By varying the height of the Soxhlet tube and the condenser the apparatus could be adapted for use with various solvents, and the temperature of the actual extracting solvent could be varied within considerable limits. Another important advantage was that all corks and joints were dispensed with, and the apparatus was easy to manipulate and clean.

Hydrolysis of the Fat.

The fat was hydrolysed in ethereal solution with a large excess of an alcoholic solution of sodium ethoxide, as described in previous papers, but as the esters of cholesterol are said to be difficult to hydrolyse in the cold by this method, the mixture was refluxed for 4 or 5 hours on the water-bath, and then allowed to stand 24 hours. The soaps were filtered and well washed with ether, or, if too large for this, extracted in the apparatus described above. The ethereal solution of unsaponifiable matter was then thoroughly washed free from alcohol and traces of soap, made to known volume, and an aliquot portion evaporated and weighed.

Estimation of Sterols Precipitable by Digitonin.

This estimation was carried out by the method described by Fraser and Gardner (1910) on a suitable portion of the solution.

Most of the experiments were carried out on human liver and spleen, obtained from cases of healthy adults killed suddenly by accidents. In these cases it was not possible to obtain the material until some 24 hours after death, during which time *post-mortem* changes might have taken place, with possible destruction of the enzymes responsible for the changes under investigation. Control experiments were therefore made on liver and spleen obtained from oxen killed at the slaughter-house by the Kosher method. The material thus obtained was treated within 2 or, at most, 3 hours after death. In one experiment pure cholalic acid was added to the autolysing tissue, to test the statement of Abelous and Soula that the addition of a small quantity of this substance would markedly increase the synthesis of cholesterol.

Our results are given in the following Tables—(I) Liver, (II) Spleen.

Discussion of Results.

In all these experiments, such aliquot portions were taken for analysis as would give between 0·18 and 0·25 grm. of digitonide for weighing. It was not possible to use larger quantities than this owing to the scarcity of digitonin, but in our experience these quantities are quite sufficient to give reliable results, other things being equal.

When we consider the large and complex series of operations necessary in carrying out these experiments the errors must be considerable, however great the care taken. We think that errors creep in mainly in the extraction process. In the Tables we give the sterol results to the third place of decimals, which would be justifiable if we were dealing with pure sterols. The relatively minute errors inherent in the digitonin method itself are, of course, magnified in calculating the quantities actually weighed to percentage of the original material taken for autolysis and extraction. In our opinion, second place may be regarded, however, as approximately correct.

A careful consideration of the figures indicates no evidence whatever of either synthesis, or destruction of cholesterol, during autolysis, and as far as

Table I.—Liver.

Source.	Weight of original organ taken for experiment.	Pre-servative used.	State of tissue.	Duration of autolysis.	Temperature of autolysis. ° C.	Ether extract.	Unsap. matter.	Sterol by digitonin.		Results.
1. Human (accident)	570	Toluene	Fresh from P.M.	—	—	3·18	0·29	0·146	Difference −0·007	
	570	,,	Autolysed	14 days	37	2·45	0·29	0·139		
2. Human (accident)	530	,,	Fresh from P.M.	—	—	6·95	0·63	0·268	Difference +0·017	
	530	,,	Autolysed	30 days	37	6·26	0·57	0·285		
3. Human (accident)	232·2	,,	Fresh from P.M.	—	—	6·37	0·79	0·257	Deviation from mean value 0·289	−0·032
	250·2	,,	Autolysed	24 hours	37	6·85	0·77	0·251		−0·038
	205·5	,,	,,	48 ,,	37	6·40	0·98	0·344		+0·055
	207·7	,,	,,	7 days	37	6·93	0·79	0·304		+0·015
4. Human (accident)	154·4	2 per cent. solution of sodium fluoride	Fresh from P.M.	—	—	4·78	0·64	0·295	Deviation from mean value 0·289	+0·006
	154·3	,,	Autolysed	1 day	37	4·67	0·63	0·292		+0·003
	161·9	,,	,,	6 days	37	4·82	0·77	0·281		−0·008
5. Ox liver (Kosher)	157·1	,,	Fresh from slaughter-house	—	—	6·89	0·59	0·284	Difference +0·013	
	155·3	,,	Autolysed	24 hours	37	6·70	0·65	0·297		

Table II.—Spleen.

Source.	Weight of original organ taken for experiment.	Preservative used.	State of tissue.	Duration of autolysis.	Temperature of autolysis.	Ether extract.	Unsap. matter.	Sterol free of digitonin.		Results.
					° C.					
1. Human (same subject as liver 3)	15·27	Toluene	Fresh from P.M.	—	37	2·31	1·43	0·366	Deviation from mean value 0·364	+0·002
	14·18	”	Autolysed	24 hours	37	3·46	1·47	0·353		−0·011
	17·70	”	”	48 ”	37	2·87	1·36	0·352		−0·012
	14·43	”	”	144 ”	37	3·43	1·31	0·385		+0·021
2. Human (same as liver 4)	29·80	2 per cent. sodium fluoride sol.	Fresh from P.M.	—	37	2·63	1·08	0·275	Deviation from mean value 0·296	−0·021
	29·95	”	Autolysed	24 hours	37	2·69	0·97	0·320		+0·024
	32·43	”	”	144 ”	37	2·16	0·99	0·304		+0·008
3. Human........	27·69	Toluene	Fresh from P.M.	—	37	2·23	0·95	0·428	Difference	−0·036
	23·35	”	Autolysed	24 hours	37	2·27	1·09	0·392		
4. Ox (Kosher)	140·3	2 per cent. sodium fluoride	Fresh from slaughter-house	—	37	2·63	0·63	0·447	Deviation from mean value 0·446	+0·001
	125·9	”	Autolysed	24 hours	37	2·07	0·72	0·439		−0·007
	174·6	”	Autolysed, with addition of 0·7 gr. of cholalic acid	48 ”	37	2·29	0·80	0·453		+0·007

autolytic experiments afford evidence on the point, these organs are not concerned with the synthesis of cholesterol in the organism.

In column 8, we give the percentages of unsaponifiable matter. These figures are probably a little too high, owing to the presence of traces of resins which we have shown (1921, Gardner and Fox) are produced by the action of alcohol on alkali during hydrolysis, but it will be noticed that the total unsaponifiable matter is two to three times the weight of sterol precipitated by digitonin. The composition of this unprecipitable portion is under investigation. With the Burchardt-Liebermann reagent this substance gives a reddish brown colour changing to green, in a manner similar to that of the portion of the unsaponifiable matter of fæces not precipitated by digitonin.

Estimations were also made of the free and ester cholesterol present in the fresh and autolysed tissue, but we do not give the figures at this stage of the inquiry, because we think that the question of the presence of ferments capable of hydrolysing or synthesising sterol esters, is better attacked by investigating the effects of tissue extract on pure cholesterol esters. We may say, however, that our results are in general agreement with those of J. H. Schültze, previously mentioned.

Conclusion.

As far as autolytic experiments are concerned, there is no evidence that either liver or spleen are the organs which have to do with the synthesis or change of cholesterol, as maintained by Abelous and Soula.

We take this opportunity of expressing our thanks to the Government Grant Committee of the Royal Society, for help towards the expenses of the research; to Dr. Schryver, for kindly supplying a sample of pure cholalic acid; and more especially to Dr. Donaldson, of St. George's Hospital, for the care and trouble he has taken in obtaining the *post-mortem* material; and to Dr. Rashleigh, for valued help in some of the earlier experiments.

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OBITUARY NOTICES

OF

FELLOWS DECEASED.

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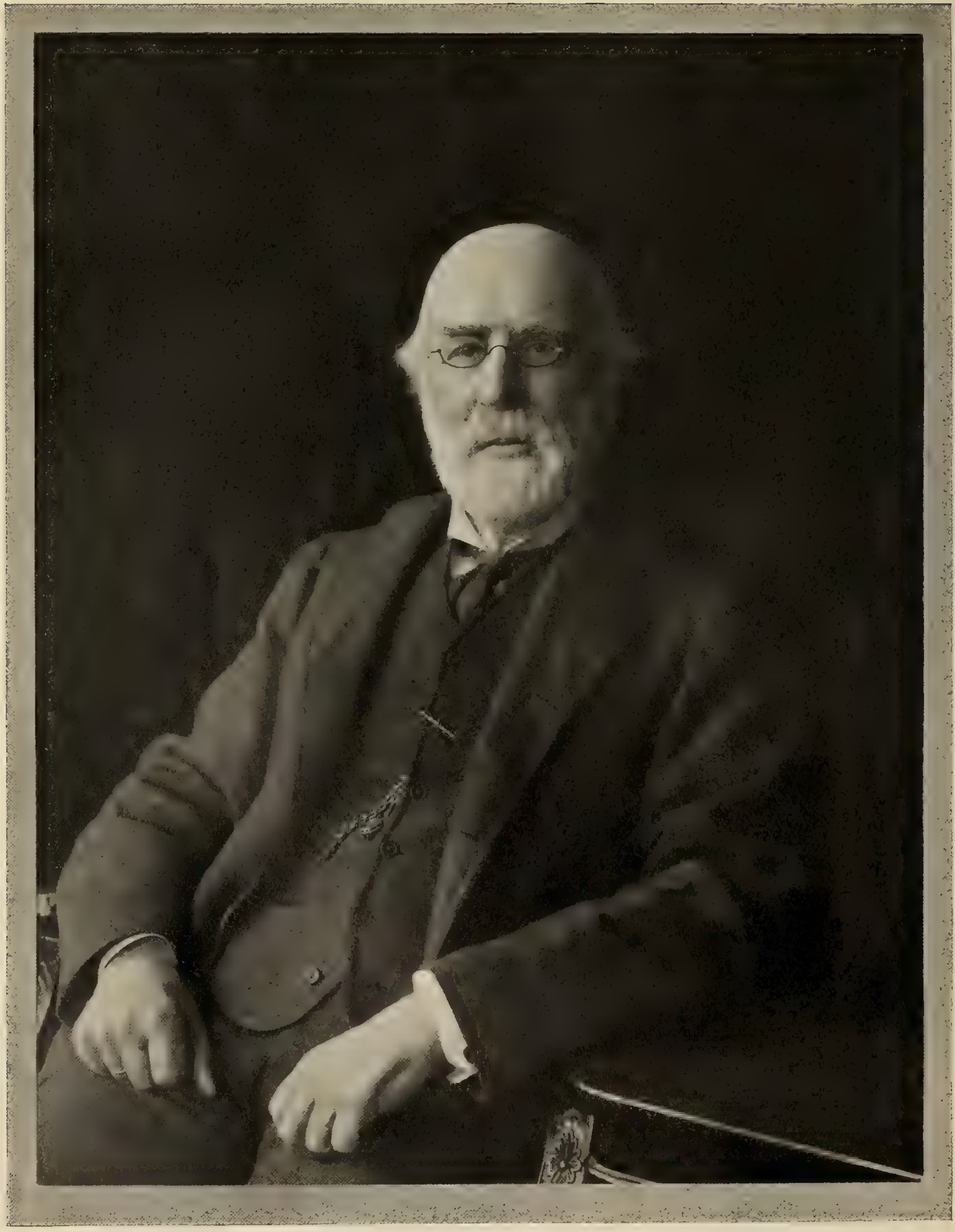


Ducie

EARL OF DUCIE, 1827–1921.

THE late Earl of Ducie, who died at his seat, Tortworth, Gloucestershire, was born on June 25, 1827, and was the "father" of the House of Lords, which he entered on the death of his father in 1853. He was also the "father" of the Royal Society, to which he was elected in 1855, as well as the oldest Lord Lieutenant in England, having been appointed by Palmerston in 1857.

He was the eldest of ten brothers and four sisters, several of whom survive him, but his only son, Lord Moreton, died in 1920, leaving no heir, so that he is succeeded by his brother, the Honourable Berkeley Moreton, formerly Colonial Secretary for Queensland, where he has resided for many years.

Lord Ducie was a type of man more common perhaps in the early Victorian era, to which he belonged, than at present, who received the honour of our Fellowship rather on account of his position and his general interest in Science, than for any special study or work that he had done.

If, however, Arboriculture is a science, and a tree-planter has a claim to be elected an F.R.S. in virtue of his lifelong devotion to a pursuit which has beautified rural England beyond any other country, then I have no hesitation in saying that he would, even as a simple country squire, have deserved it. When he inherited the noble domain of Tortworth, 68 years ago, he found nothing more to induce him to become a leader in Arboriculture than many others have found; for his father, though an eminent agriculturist, had done little or nothing to set him an example. Loudon had laid a good foundation for more exact knowledge than our ancestors possessed of the great number of exotic trees which will thrive in this country, and the introduction by Douglas and others of numerous North American conifers, had attracted many and created a fashion for planting a "Pinetum" in many parts of England. Some of the most famous of these Pineta were made on soils or in climates which later experience has proved to be unsuitable, and in consequence when the generation which had planted them died out, they sometimes became neglected and many of the species died. But the varied soils and situations at Tortworth, coupled with the favourable climate of the vale of Gloucester, favoured Lord Ducie's early experiments so well, that he never rested in his labours till he was past ninety, when his bodily and mental powers began to fail. But his pleasure and interest in watching the growth of his trees never diminished, and he set an example to all planters by the care which he gave to his trees for many years; with the result that at Kew alone can a collection be found, so well grown, well pruned, and generally well cared for as at Tortworth.

When I first began to collect material for a more up-to-date work on the trees of Great Britain, he encouraged and assisted me to a greater extent than any other man, and was never so pleased as when he could show me in one or other remote corner of the park or plantations, some rare American oak, hickory, or other hard-wood which he had planted 40 or 50 years before. Visitors were numerous at Tortworth, and though he loved to show them round himself, he was such a conscientious man in the performance of the numerous public duties which he undertook, that he always seemed to be in a hurry to get on the next tree or the next duty, so that one had to repeat one's visits frequently in order to take in the knowledge he possessed. I well remember telling him of a tree in Lord Bathurst's park which I could not identify with certainty. He said "What is the use of your knowledge if you cannot name a tree?" and wrote to Sir J. D. Hooker to send down the late Mr. Nicholson to name it; who, suspecting, as I did, that it was of hybrid origin, identified it with *Pyrus intermedia.* But it was only after my personal visit to Fontainebleau, and a week's work by Dr. Henry in the Kew Herbarium that we came to the conclusion that it must be the same as what Gay and other French botanists, many years before, had called the Service Tree of Fontainebleau, *Pyrus latifolia.*

When Prof. C. S. Sargent of Boston and the late Dr. Asa Gray visited Tortworth about 1880 they were shown three trees natives of California, which neither of them had seen alive in their own country and which will not exist in the climate of New England. *Castanea chrysophylla,* the Golden-leaved Chestnut, was one of these, and though the Tortworth specimen is rather a great bush than a tree, it has supplied seeds for years to all who asked for them worthily; for Lord Ducie was most liberal in distributing young trees from his well-managed nursery.

Though Lord Ducie was for many years a Vice-President of the Royal Horticultural Society, and was generally interested in local geology and botany, he took no particular interest in other branches of horticulture, though Tortworth is celebrated for its fruit. He was an active Volunteer from the commencement of the movement, a distinguished marksman with the match-rifle, a yachtsman who visited Norway on many occasions, until he lost his steam yacht in a fjord there. He was for some years President of the National Rifle Association, and a most indefatigable worker in all county business. Though Lord Ducie had resisted the request of numerous friends to compile or allow to be published a catalogue of his unique collection of living trees, it is much to be hoped that such a fitting memorial of his life's work may now be attempted. H. J. E.

ADRIAN BROWN, 1852—1919.

Adrian Brown was the junior member of the very remarkable amateur scientific quartette, Peter Griess, Cornelius O'Sullivan and the brothers Brown—Horace and Adrian, once active in the scientific service of brewing at Burton-on-Trent.

The amateur scientific worker is a peculiarly British product—he hates drill and grows through force of example, not of precept. He has not matured in countries where drill has been in the ascendant. Griess was an outstanding example. He spent six or seven years at the University—doing nothing as a formal student; the saying goes, that he wasted his time. Far from this—whilst he took his fill of student life, what to-day would be called his subconscious mind was clearly at work and he suddenly displayed extraordinary activity in the laboratory. His great ability was appreciated by Kolbe, his teacher, himself a man of the highest intelligence, to some extent trained here, under the late Lord Playfair, as fellow worker with the late Sir Edward Frankland, the ablest chemist of his time in the laboratory and the author of the theory of valency upon which our entire system of structural formulæ is based. Kolbe recommended Griess to Hofmann, then Professor at the Royal College of Chemistry, in Oxford Street, London—whence arose the dyestuff industry as the outward and visible sign of the great leader's activity and example as an original worker. Griess brought with him from Germany his discovery of the Diazo-compounds, one of the most remarkable in the history of chemistry, as it involved recognition of the fact, that nitrogen, up to that time regarded as an inert element, could form compounds of unusual chemical activity and extreme instability. He developed his discovery in London, until in 1862 he become assistant in Allsopp's Brewery in Burton-on-Trent. Here, until his death in 1888, he occupied an anomalous position, living a life all but apart from the brewery, an indefatigable worker, high up in an empyrean of constructive organic chemistry. Apparently, Griess did nothing in particular for brewing, beyond criticising its products; but he laid the foundation of a branch of the dyestuff industry which has since been the most remunerative of its many activities. His services were once sought by an English dyestuff firm, but the beggarly terms offered him were naturally declined and we lost an irrecoverable opportunity.

In the past scientific workers had their individual patrons who supported their inquiries; but the Allsopp firm behaved to their chemist in a way which is without parallel in the history of industrial enterprise; they seem to have gloried in having so distinguished a man on their staff, without considering the direct value of his services. His presence was testimony to their breadth of view, as well as to their liberality; they undoubtedly gained in repute from their action.

Horace Brown entered on his technical career, in 1866, at the time when Griess took up the position of chief chemist, at Allsopp's, vacated by Dr. Böttinger, father of the Dr. H. von Böttinger, recently deceased, who is noted for the part he played in the development of the German Dyestuff Industry. He had been influenced, as a lad, both by Böttinger and by Griess but his only didactic training in chemistry was a year spent at the Royal College of Chemistry, partly under Hofmann and partly under Hofmann's successor, Frankland.

Cornelius O'Sullivan went to Burton in 1867. He too had been a pupil of Hofmann, at the Royal College of Chemistry; he was one of the assistants in the laboratory at the time when Horace Brown and the writer entered as students. He accompanied Hofmann to Berlin but, after a few months, on his recommendation, returned to England to enter the service of Messrs. Bass and Co. An accomplished worker, he began the study of the mash-tun by investigating the action of the enzymes of malt (diastase) on starch; he may be said to have rediscovered maltose in the course of this work and to have established its significance as a fundamental unit in the complex starch molecule. Although others have followed in his footsteps, to the present day we remain ignorant as to the precise nature of the successive changes which the starch molecule undergoes on hydrolysis and of the number of enzymes concerned in the process. O'Sullivan was also the first to study, in detail, the rate at which cane-sugar was hydrolysed by yeast invertase. Finally, he undertook an inquiry into the products of the hydrolysis of gum-arabic and laid solid foundations which no one yet has built upon.

Adrian Brown did not enter the scientific service at Burton until 1873, when its foundations had been deeply laid: he then spent several years studying for the distinguished part he was to take in the quartette.

He was born at Burton on April 27, 1852. He came of a practical and nature-loving stock. His father, Edwin Brown, the son of a small builder, left school at an early age, to become clerk at a private bank in Burton, ultimately the Burton, Uttoxeter and Ashbourne Union Bank, of which he was manager during the last twenty-five years of his life. He died suddenly, in 1876, at the age of fifty-seven. He was an ardent naturalist, specially known as a coleopterist, with a strong scientific bent and leanings to all the sciences, particularly geology.

He is referred to in the 'Life and Letters of Charles Darwin' in a letter from Henry Walter Bates, the celebrated Amazonian traveller, to Darwin, dated October 17, 1862:—

> "Mr. Edwin Brown is manager in a large Bank at Burton. I have known him twenty-one years; he was my earliest naturalist friend. I have always looked on him as a man of extraordinary intellectual ability. I have given him my notices on Carabi. He is amassing material

> (specimens) at very great expense. He has never travelled; this is a great deficiency, for the relation of species to closely allied species and varieties cannot, I think, be thoroughly understood without personal observation in different countries."

In the memoir, by Mr. Edward Clodd, prefixed to the reprint of the unabridged edition of 'The Naturalist on the River Amazon,' published by Mr. Murray, in 1892, it is stated that:—

> "Bates was born at Leicester in 1825 and spent his youth in the district. Apprenticed to a hosiery business, he left it soon after his master died and eventually entered Allsopps' Brewery as a clerk."

Mr. Clodd adds:—

> "As often as he could he escaped from the desk to the open air, and some results of his entomologising are found in a paper on 'Coleoptera in the neighbourhood of Burton-on-Trent,' published in the 'Zoologist' (VI, 1848, 1997). Mr. Edwin Brown, who obtained him the situation at Allsopps', is referred to as the captor of several species scheduled in the paper."

Edwin Brown appears to have exercised considerable influence upon the fortunes of both Bates and A. R. Wallace. Bates became acquainted with Wallace at Leicester, where the latter was English master in the Collegiate School. Mr. Clodd tells us that:—

> "The two friends often discussed schemes for going abroad to explore some unharvested region and at last these took definite shape, mainly through the interest excited by a little book, published by John Murray in 1847, entitled 'A Voyage up the River Amazon, including a Residence at Para,' by Mr. W. H. Edwards, an American tourist."

The writer learns from Dr. Horace Brown that this book had greatly interested his stepfather, Edwin Brown, who lent it to Bates to read. The recovery of such a fragment of history will not be without interest, as giving a clue to the mental process whereby the two travellers were eventually led to the study of problems of world-wide significance.

Adrian Brown was therefore nurtured in a scientific atmosphere. He attended the local grammar school, but his effective training was at the hands of his father and, in chemistry particularly, of his elder brother, Horace. He received his special technical training mainly at the Royal College of Science, the combination of the Royal College of Chemistry, Oxford Street, with the Royal School of Mines, Jermyn Street, then just effected at South Kensington. On leaving, he became private assistant to Dr. Russell, Lecturer on Chemistry in St. Bartholomew's Hospital Medical School.

In 1873 he quitted London for Burton, to act as chemist to Messrs. Salt and Co., Brewers.

In referring to the Burtonian quartette, which Adrian Brown was the last to join, as amateur workers, the writer is not unmindful of the fact that all were brewer's chemists and therefore professionally engaged, excepting, perhaps, Griess—and it has always been thought that he has not had sufficient credit for the work he did in the brewery. None the less, the spirit in which they worked was that of the amateur of the past: they sought neither gain nor applause: love of their art was their guiding light; they were led solely by desire to explore its fields, to grasp its value, to display its beauties.

Adrian Brown remained twenty-five years in the brewery, leaving in 1899 to take charge of the newly founded Chair of Brewing and Malting at the Mason College, Birmingham. When the University of Birmingham was established, he became Professor of the Biology and Chemistry of Fermentation and Director of the School of Brewing. He died suddenly on July 2, 1919, three days after his wife.

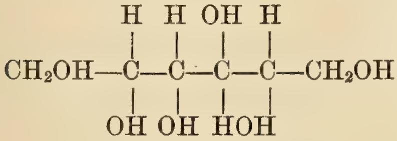
A naturalist from his birth upwards, a man of unobtrusive manner but great personal charm, he gained the esteem of all whom he met officially and the affection of his many friends. Not only was his standard of endeavour ever the highest but all he did was characterised by originality, great independence of judgment and a consistent logic.

He was not elected into the Society until 1911. His first work was in advance of the time and did not receive the attention it deserved, although the subject, the action of oxidising organisms, was one of great interest, oxidation playing so determining a part in vital activity. He began by studying the action of the well-known *Bacterium aceti,* used in producing vinegar from alcohol; then that of another organism, *Bacterium xylinum,* which he was the first to isolate from "Mother of Vinegar."

The point brought out in the earlier inquiry was the inability of *B. aceti* to condition the oxidation of methylic alcohol, although it grew in presence of this compound: then, that it was able to determine the conversion, not only of ethylic alcohol but also of propylic, to the corresponding acid; yet was without action on isopropylic and isobutylic alcohols; still, it grew in the presence of these compounds but was killed by fusel oil and amylic alcohol. These remarkable differences in behaviour of compounds so closely related remain unexplained to the present day. Assuming that oxidation be determined by a catalyst, *i.e.,* at a solid surface, it is most probable that the surface is differently affected by the different alcohols: but on any hypothesis the fact that methylic and isopropylic alcohols are unattacked, whilst compounds so close to them in all chemical properties are oxidised, is very striking; no other such marked instance of bacterial epicurism is known.

The results of his work on *B. xylinum* were of less direct significance, as his attention was mainly directed to the membrane which this organism produces in sugar solutions; he thought it was a variety of cellulose, but from later work, by Emmerling, it is probable that the product is of a chitinous character. Here again, however, a field is opened up for further inquiry—if action take place within the cell, how comes it that such a product is secreted without the organism if this be not destroyed in the process?

The activities of *B. xylinum* were studied, a few years later, by the French chemist, Bertrand, who established the fact that it has an entirely remarkable discriminative power, as in compounds sensitive to its oxidising influence two H.C.OH groups are present, not only in conjunction but so placed that the two OH groups in these are on the same side of the plane in the formula; thus when the two isomerides formed by reducing sorboses are submitted to the action of the organism



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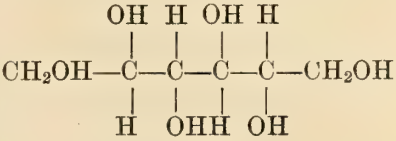
Sorbitol.



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\end{array}$$

Iditol.

sorbitol alone is oxidised and reconverted into ketose (sorbose). It is in no way clear, at present, whether the selective activity displayed by the organism be that of an enzyme or traceable merely to a peculiarity in the oxidative process.

The root ideas underlying our present conception of the nature of enzymic hydrolysis are largely traceable to Adrian Brown's iconoclastic work. Beginning with observations on the rate of reproduction of yeast cells, he noticed that a constant amount of yeast fermented an approximately *constant weight* of sugar, in unit time, *in solutions of varying concentration;* the graph of his experiments was a straight line, not a logarithmic curve—indicating the change of regularly diminishing amounts in successive unit periods—such as was held to be expressive of the simple enzymic change conditioned by invertase, on the basis of the experiments made by Cornelius O'Sullivan and Tompson, published in 1890. Hence he was led to re-examine the evidence adduced by these chemists, in support of their view, that the enzymic change was a mass-action effect, strictly comparable with the changes taking place in solutions of crystalloids—in other words, that enzymic change took place in solution.

He dealt with this subject in an exhaustive manner. The conclusion arrived at was, that during the earlier period of change, as in fermentation, the sugar is hydrolysed at a linear rate, the amount converted being practically independent of the concentration of the solution, in no way proportional to it. The rate of hydrolysis is much reduced by the addition of invert sugar, that is to say, of the products of change; lactose, except in very large proportion, however, has little effect.

These conclusions have been fully confirmed by later inquiries. It may now be taken as established, that enzymic action is effected at solid surfaces. Complete confirmation of this explanation has been given by recent observations on the action of a catalyst such as finely-divided metallic nickel in determining the hydrogenation of the fatty oils.

Much of Adrian Brown's work was in criticism of Pasteur's findings, especially the great Frenchman's conclusion that fermentation was life without air; he thought that he had proved the contrary. He studied the effect of alcohol and found that it greatly retarded the reproductive growth of yeast; also the effect of carbon dioxide but came to the conclusion that, as it had no greater influence than hydrogen and as there was a much larger increase in presence of air, that the repression of growth was due to exclusion of oxygen. He was, therefore, led to favour the conclusion that reproductive growth of the yeast cell, under ordinary anaërobic conditions, is determined by the amount of oxygen at the disposal of the organism prior to the commencement of reproduction.

Recently, however, Slator, working in the laboratory of Messrs. Bass and Co., has adduced proof that not only has carbon dioxide a greater influence than has been supposed on the activity of the yeast organism but also that oxygen is not required for reproductive growth of the cell—thereby upholding both a common opinion of carbon dioxide and Pasteur's view as to oxygen being unnecessary.

It remains to consider what is undoubtedly his most remarkable work—that on diffusion into the barley corn, noteworthy both on account of the beauty and delicacy of the method he developed and the significance of the results. Having eyes to see as well as an inquiring mind, he was led to take special notice of the blue layer just below the skin in certain varieties of barley. Desirous of finding out what happened, in the malting process, when barley was steeped in water, as it is during the preparation of malt, also what would be the influence of impurities in the water, he first studied the behaviour of dry barley-corns in water and then in various solutions. He saw that in blue barley he had a perfect mechanism for the quantitative study of diffusion phenomena. The blue layer furnished the discriminating membrane; the finely granular mass of starch within the corn served to attract water into the grain. By placing a set of weighed corns in water and at intervals removing them and determining the increase in weight, at various temperatures too, the rate at which water entered was easily ascertained. The variation in the rate at which it accumulates in the grain, as the temperature is raised, was in agreement with that at which the vapour pressure of water rises when this is heated.

A similar tale is told by solutions of most salts and of substances such as the sugars—these all have vapour pressures lower than that of water and water accumulates less rapidly in barley corns placed in these solutions than if they were in water, the rate depending on the concentration.

Solutions of the ordinary strong acids and alkalis also give up water to dry barley corns and become concentrated but no acid passes across the discriminating membrane; the blue colour remains unchanged in all sound corns.

But weak acids, also weak alkalis, such as ammonia, readily pass through; moreover, the membrane is penetrable by all chemically neutral substances at all soluble in water—such as acetic acid and its homologues, chloroform, even hydrocarbons such as benzene. These all pass into the corn together with water and actually accelerate the passage of water into the grain. It is no question of molecular size: such molecules do not penetrate the membrane because of their smaller size; on the contrary, butyric acid enters more rapidly than acetic and the alcohols of the ethylic series pass through the more rapidly the greater their molecular weight, so long as they are reasonably soluble in water.

Eventually an equilibrium is reached within the corn and the concentration of the solution may become higher than in the liquid outside; it has even been observed, in the case of phenol and aniline, that the internal solution is "supersaturated." To take an example, when the corns are saturated in a solution containing 50 per cent. of acetic acid, the solution within contains 80 per cent. of the acid—but this is the limit, no more passing in from stronger solutions.

Previous observers on the passage of such substances through living tissues have correlated their relative activity and their lethal power with the solubility in fats, and have postulated the existence of a lipoid layer at the tissue surfaces. Adrian Brown's observations justify us in putting aside all such fancy explanations—the correlation is of consequence only in so far as the solubility referred to of substances in oils and fats is usually the converse of their solubility in water.

A full discussion of the work Adrian Brown accomplished and its bearing on contemporary inquiries, by the writer, is published in the 'Journal of the Institute of Brewing,' 1921, vol. 27, pp. 197–260. From the point of view advocated in the present notice, his exemplary career and achievements merit most careful attention. His genius lay not on the surface but was manifest in a continuity of effort which, in sum, was remarkably effective. His work was an expression of himself: it came from within; but that he was induced, if not forced, to display his genius owing to the influence of the conducive environment in which he was placed is probably a not unwarrantable conclusion. Chamber music such as the Burtonians have discoursed so successfully might well and should be more cultivated, not only in industry but in the new Universities, even at Oxford.

H. E. A.

LOUIS COMPTON MIALL, 1842—1921.

Louis Compton Miall was born at Bradford in the year 1842, and was the fifth living son of a Congregational Minister, the Rev. James Goodeve Miall. His mother was Elizabeth Symonds Mackenzie. The teaching element was strong on both sides of his family. The Mialls had been schoolmasters and preachers for generations. One of them, Moses Miall, had published a book of Practical Remarks on Education. The Mackenzies were also much given to schoolmastering, and had come strongly under the influence of the Edgeworths, whose educational methods were firmly established in the family tradition. Sir Morell Mackenzie and Edward Compton, the actor, were first cousins of L. C. Miall on the Mackenzie side, and Edward Miall, M.P. for Bradford and Editor of the 'Non-Conformist,' was his father's half-brother.

The Rev. J. G. Miall was a man of varied attainments, and had a distinct gift for teaching. He bestowed much pains on the training of his children, for he knew that they would have to fight their way in the world on their own resources. He was very particular to train them in self-reliance, to give them studious tastes, and the power of expressing themselves well. He had himself a beautiful speaking voice, which the children all inherited in varying degree, and he taught them to make the best use of it.

L. C. Miall owed much also to his mother. She, like her husband, had a pleasant voice and manner and much charm of personality, and she had a power of holding her children's love and admiration which far exceeded his. She was, above all things, deeply religious, but had so courteous and sunny a disposition that religion, even the terrible early-Victorian religion, could not make her gloomy.

Louis seems to have been an enterprising and high-spirited child, keen about games and mischief, and inclined to wander away from home on solitary expeditions. He was sent first to a little school near his home, and, later, when he was 9 years old, to Silcoates School, near Wakefield, then, as now, a boarding school for the sons of Non-Conformist Ministers.

At the time that he left school, L. C. Miall's interests were all classical and literary. He had learned little mathematics and no science, but had shown himself good at essay writing, and had stored his mind with fine passages from Shakespeare and the Latin poets. He said in after life that he knew nothing of Natural History as a schoolboy, though he tried to make friends with a half-witted boy who could show him, as if by magic, all sorts of strange nests and creatures in places where no one else would see anything. But even in those days he must have been unusually observant, for he tells in the "Natural History of Aquatic Insects" how he and his companions watched the dragon-fly escape from its pupa-case, and how they saw the larva, "with its dingy colours, its forbidding shape, and its predatory habits . . . stretch out its great paw and secure an unsuspecting victim."

In 1857 Louis Miall left school, a boy of fifteen, but looking older, and already grave and dignified. He had probably learnt all that Silcoates had to teach him, and his father could afford to spend no more upon his education, but recognised the lad's ability, and, being anxious to give him further opportunities to study, hit upon a scheme that seemed promising from many points of view. This was that Louis should keep a little day-school, with his father's help and direction, prospectuses for which were accordingly issued in Louis's name. A member of the congregation, who much admired the minister and knew something of his teaching capacity, had already entrusted him with her son's education, and other pupils were soon found to make the nucleus of a small school. The time-table was specially arranged so that Louis should have leisure for private study; his father apparently took a good deal of the teaching into his own hands, and his mother helped with the French, and, altogether, the plan seemed to work out very well. It had its drawbacks, however, the chief of which was that Louis had to study by himself, for, though his father could help him with Latin and Greek, the boy had then no great inclination to continue his classical reading, and was more interested in the new scientific subjects that were attracting the attention of the younger generation.

His eldest brother was a medical student at Edinburgh and Louis often envied his opportunities. Probably it was his example that induced Louis later on to take a course of anatomy at the Leeds School of Medicine. It meant early rising every day to journey from Bradford to Leeds, but the teaching was good and constituted the only training in science that he could obtain.

Meanwhile he was working hard at zoology and geology, and joined a Botanical Society at Todmorden, making many friends who were interested in Natural History and publishing papers in various periodicals. One of these brought him the following letter from Charles Darwin:—

Down, Bromley, Kent.
January 23, 1860.

DEAR SIR,

I hope that you will excuse the liberty I take in writing to you and requesting a favour. In the 'Annals of Nat. Hist.,' vol. 15, p. 39, you remark "The variations of form in the maxillæ are of no value among the Phalangida in affording generic or specific characters as with the true spiders." Am I to understand from the latter part of sentence that with the individuals of the same undoubted species the maxillæ vary in form? Is not this a very surprising fact? Would you have the great kindness, if the fact be so, to give me some details on the amount and kind of variations and in what species. And further would you permit me to quote any such facts on your authority?

With many apologies for troubling you, I beg to remain, Dear Sir,

Yours very faithfully,
CHARLES DARWIN.

One wonders what answer was sent, and whether Darwin was aware that his correspondent was a boy of eighteen.

A couple of years later appeared the "Flora of the West Riding" by Miall and Carrington, with an introduction by Louis Miall, which he repented in later years; for, though it has merits, it is written in a rather high-flown style, and to publish a list of plants and their localities was quite contrary to his maturer teaching.

Besides the difficulty of studying without teachers, there was another drawback to the life that he was leading at this time. His father was strong-willed and autocratic, and Louis' own strong will was frequently at variance with his in the management of the little school. The young man's scientific studies, and the spirit of the age when Darwin and Huxley were fighting for freedom of belief, soon brought religious disagreement into the family circle and Louis' change of faith was a great grief to his parents. It hit his father on all sides, as parent, schoolmaster and minister, and he felt it very bitterly. Altogether the life was neither happy nor hopeful, and the young man decided that it could not continue. He would find work elsewhere, and eventually he took a post as assistant-master in a school kept by Mr. George Todd at Stamford Hill, near London.

Towards the end of the second year there the situation was changed by a letter from his brother Philip, telling him that a Philosophical Society was being started in Bradford, and that Philip was commissioned to write and offer him the post of Secretary to the Society with a salary of £100 a year. This was just what he wanted. He wrote an immediate acceptance and gave notice to leave the school at the earliest moment possible.

This was the turning point in Louis Miall's career. After six or seven years of gradually increasing darkness and discouragement, the horizon cleared, and henceforth he advanced without faltering. When he returned to Bradford he was very raw and inexperienced and had little idea what to make of his new task. The first thing he had to do was to arrange a course of lectures, under the guidance of the Committee, who soon left all the correspondence in his hands. An interesting course of lectures was given between 1865 and 1871, among others by Owen, Huxley and Rolleston, who thus came into personal contact with the Secretary of the Bradford Philosophical Society.

Another thing to which the Secretary had to turn his attention was the making of a museum from a collection of objects mostly given by people who wanted to get rid of them. He finally decided that the only thing he could do was to make a collection of geological specimens for which the neighbourhood offered unusual facilities. He prepared a report to the Committee, in which he offered to collect what he could from the coalfields and limestone districts within reach. For some years it was his delightful hobby to explore the district of Craven, to study its geology and to collect its fossils. A frequent companion of his on these rambles was John Brigg, afterwards Sir John Brigg, M.P. for Keighley, a member of his

Committee, who took a great interest in the young Secretary and influenced him in many ways.

In the course of these excursions quite a respectable collection of fossils and geological specimens was made for the Bradford Museum. Then a great piece of luck befel the Curator. One day there came into his office a coal miner bringing some curious bones that he had found in the Low Moor coal mine. Miall went to see them himself next day, going down a coal mine for the first time in his life. He was shown the bones on the roof of a passage in the works, and realised that they would require very careful treatment if they were to be removed without injury. So it was decided to apply a layer of plaster of Paris to protect the bones and then to have the coal carefully worked away, a prop being placed to support the fragments covered with plaster. The bones were removed in perfect condition except for those that had already been broken off. The block removed was 11 feet long and a couple of feet wide. Investigations proved that the bones belonged to a Labyrinthodont of a species that was hitherto unknown. On the suggestion of the Committee, Miall wrote to Prof. Huxley and offered to take the fossil to London and show it to him. Huxley sent an encouraging reply, the fossil was carefully packed in a wooden case and taken to London, where it was examined with much interest by Prof. Huxley and Prof. Flower. Huxley undertook to write a description of it for the Geological Society and asked Miall to prepare a short account of its discovery and removal from the coal mine. At the next meeting of the Geological Society, Miall read his paper and Huxley gave a simple and interesting account of the new Labyrinthodont, without notes, explaining it from the specimen as he went along. Sir Charles Lyell was present and seemed to be much interested.

When Miall returned to Bradford and gave the Committee of the Philosophical Society a vivid account of what had passed, they asked him to repeat the story in the form of a lecture to the Society. It was his first public lecture. After spending a good deal of time trying to write it out, he resolved to follow Huxley's example and speak without notes, explaining the actual specimen before the audience. There was a good attendance, for the matter had aroused interest in Bradford, and the lecture went off very well. That was the beginning of Miall's career as a public lecturer. After that we find him giving courses of Lectures in Bradford and Leeds mostly on Geology, but also on Botany and the "Early History of Domestic Animals."

Though very shy and studious, Miall seems to have entered somewhat into the social life of Bradford, which happened to be unusually interesting at that time. He was fond of music, and indeed had studied it in his usual way by sheer force of will and without a teacher, so that he had written songs for his sister and could play to some extent on two or three instruments. He also had a good deal of talent for painting. He brought back from a cruise in the Hebrides in 1868 sketches from which he made some clever little water-colour pictures, that still hang beside one or two of his father's in homes of a younger generation.

At Bradford he met his future wife, Emily Pearce, to whom he was married in 1870. Though not scientific, her intellectual and social gifts were, in some directions at least, equal to his own.

In 1871 L. C. Miall was appointed Curator to the Leeds Philosophical and Literary Society. He had already delivered a course of lectures on geology to the Society and was known to several influential people in Leeds. He must have had Huxley's support, too, in his application, for among his letters of congratulation on obtaining the post was one from Huxley, in which he characteristically remarks that it would be a matter of great satisfaction to him to think that he had in any way contributed "to the putting of an indubitably square man into the square hole at Leeds."

His interests at this time were mainly geological, and he devoted himself to the collection of fossils in the Leeds Museum with the same enthusiasm that he had given to the geological collection at Bradford. He was helped in its re-arrangement by Pengelly, Boyd Dawkins, and others. Later, much help and many specimens were given by A. H. Green. When it was re-arranged, he wrote a guide to the collection; in the same way he re-arranged the different collections of birds, insects, antiquities, and so forth, and wrote a guide to each, in which he set forth clearly the general principles of the various subjects.

Since 1869 Miall had been busy with the investigation of the new Labyrinthodont that had been found in the Low Moor coal mine. The task proved more difficult than he had expected. He was Secretary of the Geological Section of the British Association at Edinburgh in 1871, and a Committee was then formed, consisting of Phillips, Woodward, John Brigg, and three others, with Miall as Secretary, to investigate and compare all the known species of Labyrinthodont. It happened that the following summer John Brigg and his friend, Swire Smith (Sir Swire Smith, whose life has been written under the title of 'The Master Spinner'), decided to go to Germany to look into the German system of education and see for themselves how far such a system would be possible in industrial England. They invited Miall to join them, so that he and John Brigg could combine the investigation of Labyrinthodonts with the educational work, all three being in fact interested in both subjects.

They had an instructive tour, and the following year (1873), when the British Association met at Bradford, Miall read the report of the Committee on Labyrinthodonta. The work had been very thorough: "Some of the members have personally examined all the more important Labyrinthodonta in European collections, including at least one example of every species recorded from the British Isles." The report created much interest and brought Miall into general notice for the first time.

Miall was now beginning to concentrate his attention on Biology. He declined the Professorship of Geology at the newly opened Yorkshire College in favour of A. H. Green, a much stronger geologist than he felt himself to be, and henceforth his interest in geology began to wane. He never cared greatly for mere collection and the minute characteristics of the shells in which animals had lived. He collaborated with A. H. Green, Thorpe, Rücker, and Marshall in a work on 'Coal, its History and Uses,' published in 1878, but his serious interest in geology and palæontology ended about the year 1880. He never studied petrology, without which much of the recent work cannot be appreciated.

When Miall refused the Professorship of Geology, the Council of the Yorkshire College still wished to secure him upon its staff, and appointed him the following year (1875) lecturer in Biology, a post which he held concurrently with his curatorship of the Museum. In 1876 he was made Professor of Biology. Many of his lectures were given in the library of the Philosophical Society, for the Yorkshire College had little accommodation, while there was room and a store of material at the Museum. The professors of the Yorkshire College frequently gave lectures to the Philosophical Society and sat on its Council. Both institutions worked in conjunction with the Leeds School of Medicine, which required courses of Botany and Zoology for its students.

It was in the yard of the Medical School that Miall dissected the Indian elephant which chance gave into his hands. A shed was built over the animal, and there he worked through the cold winter of 1874–5, helped by F. Greenwood, Curator of the Medical School. The memoir on the "Anatomy of the Indian Elephant" appeared in 1879, and was the second of a series of studies in comparative anatomy. The first of the series was the "Skull of the Crocodile," which appeared in 1878, and the third was the "Structure and Life-History of the Cockroach" (1886). There the series ended abruptly, for though a short account of Megalichthys, a ganoid fish of the Coal Measures, was published in 1885, the fourth book of the series, which was to have dealt with that topic, was never written. The author had given so much time to the Cockroach, and had become so deeply interested in it, that all other research had to give way to the structure and life-histories of insects, which occupied him as long as he had vigour and eyesight for the work.

The book on the Cockroach, published in conjunction with Alfred Denny, was by far the most important piece of work that Miall had done so far. It represented several years of study, begun in the Museum of the Philosophical Society, and carried on at the Yorkshire College and at his own home. It has since been recognised as marking an epoch in the study of insects in this country. In reading up the subject as a preliminary to further research, he had become acquainted with the work of the old naturalists, Malpighi, Swammerdam, Lyonnet and Straus-Durcheim. He found them so fascinating that the first chapter in the "Cockroach" is devoted to them, and the whole book is an exposition of their teaching—a very lucid account of insect structure and development. Its value was immediately recognised by Prof. Huxley who congratulated Miall on the book.

The "Cockroach" appeared in 1886. In 1887 we find its author already occupied with another insect, Chironomus, the Harlequin Fly. This was chosen because of its abundance nearly all through the year, its transparency (in contrast to the Cockroach), and the ease with which it can be reared. Besides which, he says, Chironomus, in its various stages, has a very special biological interest. His attention was concentrated upon it for several years. The " Structure and Life-History of the Harlequin Fly," by Miall and A. R. Hammond, did not appear till 1900, though most of the work was done by 1892.

Soon after he began work on Chironomus, Miall visited Leyden to consult some books there. Every letter of this period has some reference to Chironomus, and we even find him " reading Dutch for the sake of Chironomus "; but, nevertheless, he found time to write on educational topics in the 'Journal of Education' and to devise " Object Lessons from Nature," which appeared in book form in 1891.

Nature-study had not at that time become a universal subject of school teaching, but object lessons were given habitually by many teachers. The " Object Lessons from Nature " were intended to emphasise the value of natural history in furnishing object lessons for children. In 1878, a course of nature object lessons to children had been given at the Museum of the Philosophical Society, under his direction, so that the idea was not a new one to him.

A natural development of this was the Saturday morning class for teachers which was so valuable a feature of the Biological Department for many years. The school-masters and mistresses came at first with the idea of getting up a few object lessons for their schools, but eventually many of them came year after year from love of the work, and were the most enthusiastic students that attended the Department. It was a considerable tax on the energy of the staff, and Miall was fortunate in having the hearty co-operation of all concerned. A further extension of this work with teachers took the form of three summer courses in nature study, given in 1901 and the two following years, at Berwick, Rothbury, and Hexham. Here, again, he had the help of his staff, and all looked back with pleasure on the experience.

The investigation of Chironomus led to that of aquatic insects in general, and, in 1891, Miall gave one of the public lectures to the British Association at Cardiff on " Some Difficulties in the Life of Aquatic Insects," treating specially their means of overcoming the surface tension of water. He also read a paper on floating leaves in connection with the same difficulty. A piece of work on Transformation of Insects, which appeared in " Nature " in 1895, was also a product of the Chironomus investigation, and that year, five years before the book on the Harlequin Fly was ready, Miall completed the " Natural History of Aquatic Insects," a semi-popular book on the subjects that he was studying. As in the " Cockroach," he draws attention to the work of " certain old zoologists—Swammerdam, Réaumur, Lyonnet, and De Geer—who are at present unjustly neglected." " Some passages in this book," he says, " if taken alone and read hastily, may appear to disparage systematic zoology. This is far from my intention. No one can study the great naturalists of the seventeenth and eighteenth centuries without feeling how seriously their work is impaired by the defective systems of the time. It is not systematic, but aimless work that I deprecate—work that springs from no real curiosity about nature and attempts to answer no scientific questions." The book was illustrated by A. R. Hammond, who collaborated with Miall in the production of the "Harlequin Fly," and made most of the beautiful illustrations for that work also.

In 1892, Miall's many preoccupations obliged him to give up the Curatorship of the Philosophical Society, though he still continued to serve on its council. About this time he left Leeds and went with his wife to live at Ilkley, as their children were all scattered for the moment. He subsequently took a house at Ben Rhydding, where he wrote "Round the Year," a series of nature studies, in some respects the most memorable book that has appeared from his pen. He was by this time 54, and henceforward undertook no new work that involved much close microscopic investigation, such as he had given to the Cockroach and the Harlequin Fly, but devoted himself rather to general topics of natural history and to educational work. "Round the Year" may almost be regarded as a piece of literature; it has been compared with Gilbert White's Letters and was written in the same spirit, not as work, but as a pleasant relaxation in the twilight of a busy day. It led to the study of Gilbert White, and to the preparation of a new edition of the Natural History of Selborne in conjunction with Dr. W. Warde Fowler. It was followed, in 1904, by another book of the same kind, "House, Garden, and Field," which has not quite the freshness of "Round the Year," and was meant partly to satisfy the teachers who were clamouring for more object lessons. The author thought it would be better if they made their own lessons, and that nature study could not be taught effectively by those who lacked time or inclination to do so, but he was quite willing to suggest topics for those who cared to develop them.

In 1897 appeared "Thirty Years of Teaching," which embodies his experience in various kinds of teaching, including the education of his own children. A good deal of it had been printed in the "Journal of Education," and was written in the train going to and from Leeds. The most important feature of the book is the method of treating University or College students which it advocates—a method not indeed new, except as applied to them.

When the British Association met in Toronto in 1896, Miall was President of Section D. His address to the section was an eloquent plea for studying *life*, the modes of growth of individuals and races, the causes of decay and extinction, and the adaptation of living organisms to their surroundings. "The animals set before the young zoologist are all dead; it is much if they are not pickled as well," he complains, and he asks why we study animals at all, giving various answers to the question, but ending "to know more of life is an aim as nearly ultimate and self-explanatory as any purpose that man can entertain." Furthermore he urges the historical method of treating various biological subjects, and shows how much keener interest can be aroused in such a topic as the Alternation of Generations by finding out step by step how it was discovered, and sharing the discoverer's own enthusiasm, than by taking it as a mass of cut-and-dried facts.

After spending half a dozen very pleasant years at Ben Rhydding, Miall moved back to Leeds, partly for the convenience of the two sons who were then at home again, and he remained in Headingley till he gave up his Professorship in 1907. The last years in Leeds were much occupied with methods of teaching, and he now attended the new Education Section of the British Association when he happened to be present at the meetings. In 1903 he was chairman of a committee to report on the teaching of Botany.

In 1902 appeared a volume on "Injurious and Useful Insects," an excursion into economic entomology, which he felt to be an important field of investigation, needing especially complete life-histories of insects to make it valuable. No doubt the main idea was right and has since been followed up with good results, but Miall was not himself in close enough touch with agriculture to make the book altogether a success, from the economic point of view. The life-histories of insects that it contains are, however, still useful to economic entomologists.

At the inauguration of the University of Leeds in 1904, Miall was given the Honorary Degree of D.Sc., the only academic distinction that he ever attained. That year and the year following he had the honour of holding the Fullerian Professorship at the Royal Institution. At that time also he was asked to serve on the Council of the Royal Society, but unwillingly declined as he had already so much on hand.

In 1908, after his retirement to Letchworth, he was President of the Education Section at the British Association in Dublin, and that was the last meeting that he was able to attend, on account of increasing deafness. Many activities had to be given up for the same reason, but he was still able to carry on individual teaching. From his wife, who was as keen an educationist as himself, he had learnt the direct method of teaching modern languages and applied it in a way of his own to the teaching of Latin, writing out a series of oral lessons and learning, when over seventy, to speak Latin fluently with the modern pronunciation. Since his school days, he had never altogether neglected his classical studies and, though he sold most of his books when he left Leeds, he had kept such Latin and Greek authors as he happened to possess.

It might be noted here that all his life he loved books and was interested in the care and binding of them. He was for many years Hon. Librarian of the Yorkshire College. The only half-disparaging remark he was known to make about Charles Darwin referred to the ruthless way he treated books.

Writing was an occupation that he maintained to the end of his life. The first book that he wrote at Letchworth was the "History of Biology," a clear and illuminating résumé of the subject that led to the more important work on the "Early Naturalists (1530–1789)." It begins with an introductory chapter on Natural History down to the 16th century, and consists mostly of biographical sketches of the old naturalists he loved so well, but there are also digressions on "The Natural History of Other Lands and the Investigation of the Puss Moth and of the Flower." Of this book Dr. Warde Fowler remarks: "He fairly astonished me, after a visit here at Kingham, by sending me as a gift the five splendid volumes on insects of Réaumur, and later on his own book on the 'Early Naturalists,' one as great a treasure as the other, for his own beautiful English was as clear and enjoyable as Réaumur's French."

Miall's great force lay in his absolute sincerity. Though he could write well, and even brilliantly, he never wrote for effect. Everything that he published represented all the careful research and investigation that the subject demanded. His first attempts at solving a problem were usually wrong, he tells us, and in regard to one of his later books he says that every time he looked up a fact in the British Museum, he found two fresh ones that required investigation. "Fortunately," he adds, "I am not pressed for time."

"The Early Naturalists" was the last book he published. He spent some years on "A History of Garden Craft" which was ready for publication when the war broke out in 1914, but was then put aside, and after that he wrote no more books. He wrote an occasional paper, carried on a correspondence (sometimes in French) with one of his brothers, and made letter-writing rather a hobby. Gardening had long been a hobby of his and he had given a good deal of attention to the laying out of his new garden at Letchworth.

On the death of his wife in 1918, my father came back to his favourite haunts in Ben Rhydding and remained there till his last illness. He died on February 21st, 1921, at our house in Leeds. By his own wish there was no religious service at his funeral, a few words of farewell being spoken by his friend, Prof. Smithells, in the presence of a small gathering of relatives, old friends and colleagues. Nevertheless, the religious enthusiasm which inspired his early manhood had never altogether left him, his attitude to life and the unknown was always reverent, and the influence he exerted on those among whom he worked was spiritual as well as intellectual.

W. W.

GEORGE STEWARDSON BRADY, 1832—1921.

G. S. Brady, M.D., M.R.C.S., D.Sc., LL.D., F.R.S., C.M.Z.S., Professor of Natural History, Armstrong College, Newcastle-upon-Tyne, and Consulting Physician to the Sunderland Infirmary, was born, he told me, April 18th, 1832. Presumably also on his authority we learn that the event occurred at Gateshead, and that he was the eldest son of Henry Brady, surgeon.

As his childish education began at the Friends' School, Ackworth, it is not improbable that he owed the name Stewardson to his parents' acquaintance with the Quaker family which gave the popular portrait-painter of that name to the early part of the nineteenth century. Certainly the whole tenor of Brady's life seems to have been in tune with the principles of that peace-loving community, and even on the scientific side there are many indications that friendship was his delight. It has been already explained in 'Nature' (January 5th, 1922), among other details, that he became a member of the Tyneside Naturalists' Field Club in 1849. At that early period it is said that his interest was "with algæ and other plant groups." Much later on he referred to these studies when pointing out in correspondence (November, 1902), that the organisms which I had described as gland-cells in the amphipod genus *Urothœ*, were, in fact, "parasites, probably algæ."

With the Natural History Society of Northumberland, Durham, and Newcastle-upon-Tyne, of which the Tyneside Field Club was a branch, Brady had a long and distinguished connexion, both as a frequent contributor to its 'Transactions,' and twice President of the Field Club. The respect felt for him by fellow-workers in systematic zoology may be partially traced by the use of his name in classification. Thus among Copepoda Axel, Boeck names a genus *Bradya* in 1872, Thomas Scott supplies *Neobradya* in 1892, Giesbrecht *Bradypontius* in 1895, and *Bradyidius* in 1897, Vanhöffen *Bradyanus* in the same year, and G. O. Sars *Pseudobradya* in 1904. Sars had named a genus *Bradycinetus* in 1865. But this suggests a curious need for caution in that many generic names owe the commencing syllables Brady-, not to eminent zoologists, but to the Greek βραδύ, indicating some organic slowness, and very inappropriate to the scientific activities of George Brady and his brother Henry. For the use of the former's name in identifying species, his friend A. M. Norman led the way with the Ostracode *Cythere Bradii* in 1864. But this, for technical reasons, gave way to another species, the Marquis de Folin's *Cythere Bradii* in 1869. Norman, in 1878, named a Copepod *Cervinia Bradyi*, Sars in 1884 another of that group *Undinopsis Bradyi*, and Thomas Scott a third in 1892 as *Tetragoniceps Bradyi*, but this, later on, he found reason to place in a new genus with the long-flowing name of *Phyllopodopsyllus*, strictly meaning "a leaf-footed flea," the species being notable for "the large size and leaf-like form of the fifth pair of thoracic feet of the female." In a footnote to *Tetragoniceps Bradyi*, Dr. Thomas Scott

George S Brady

remarks, "the name is given in compliment to Prof. G. S. Brady, who instituted the genus, and to whose untiring and disinterested kindness the author of these notes owes much of his success in the study of the Entomostraca." In 1879 Dr. Norman again pays his friend the compliment of using his name for a species, this time in the eccentric group of the Sympoda, to which he adds the description of *Diastylis Bradyi.*

In the previous year the Ray Society had published the first volume of Brady's "Monograph of the free and semi-parasitic Copepoda of the British Islands." As the uninitiated may be excused for wondering why men of ability should spend a considerable part of their lives in studying creatures so insignificant in size and so generally harmless to mankind, as the Entomostraca, it may be observed that, as in old Camden's phrase, "many a little makes a mickle," and as little grains of sand may make a mountain, so the stupendous multitudes in which some of the entomostracan species occur make them indirectly yet ultimately important contributors to human food and comfort. But, apart from economic values, the true lover of nature finds in this seemingly trivial study more than one source of æsthetic fascination. In the introduction to Brady's last-mentioned work he says:—"Some of the pleasantest and most profitable hours which I have ever spent have been when, after a day's dredging, I have set out at sunset on a quiet boating excursion for the purpose of capturing such prey as could be got in the surface net. Many hours of this kind, spent in the company of my old friend Mr. David Robertson, amongst the Scilly Islands, on the Firth of Clyde, on the sheltered bays of Roundstone and Westport, or on the stormier coasts of Northumbria, will long live in my memory, not only by their results in the acquisition of valuable specimens, but as times of unalloyed delight in the contemplation of nature under a different guise from that in which we usually see her." The David Robertson to whom he here alludes, otherwise known as "the Naturalist of Cumbrae" (see his 'Life by his Friend,' 1891), began a notable career as a penniless herdboy, and ended it an Hon. LL.D. of Glasgow University.

In the bibliography to his luminous work on the Ostracoda of the Bay of Naples and the adjacent seas (1894), G. W. Müller enumerates twenty-one contributions by Brady to this branch of Carcinology, together with seven others in which his was the leading name in a collaboration. Five of these were undertaken with David Robertson, one with Norman, and one with Crosskey and Robertson together. When the first volume of the "Challenger" Reports on Zoology was published in 1880 under the editorship of Sir C. Wyville-Thomson, Brady was already a recognised authority on the Ostracoda. He was among those specially consulted as to the disposal of the vast "Challenger" material, and his was the third memoir to appear. It was illustrated by forty-four quarto plates. For the comparative fewness of new species he explains that the "work of the 'Challenger' gave us no collections whatever from between tide marks, nor from the laminarian zone, and these two zones usually swarm with microzoic life of all kinds." A later work of much importance was that which he carried out in partnership with Canon Norman on "The Marine and Freshwater Ostracoda of the North Atlantic and of North-Western Europe," the first part appearing in 1889, the second in 1896. In this he gives a signal example of his scientific ingenuity which is worthy of additional record. He points out (p. 622) that "In consequence of the small size of Ostracoda it is extremely difficulty to procure spirit-preserved specimens from the deep sea, and although the *Myodocopa*, being much larger than the *Podocopa*, would be detected by the experienced eye of a Carcinologist who had studied them, yet the Zoologists usually attached to Government Expeditions cannot be expected thus to notice them. Hence it is that in a large number of cases the only examples which have come into our hands are such as have been picked out of dried material. It struck us that, notwitstanding their dried condition, it might yet be possible by maceration to get some idea of the withered inmates of the shells. We therefore made experiments, and succeeded in restoring the animals beyond our most ardent expectations. All the portions of the animals figured [in several genera and species mentioned] have been taken from dissections of animals which have been preserved in a dried state for very many, in one case, as long as twenty-three years, and we are satified that these drawings will be found to be almost as exact, so far as they go, as those taken from spirit-preserved examples."

In 1884, when the editing of the "Challenger" Reports had passed into the vigorous hands of John Murray, the eighth volume of Zoology appeared, having as its opening treatise Brady's Report on the Copepoda illustrated by fifty-five carefully drawn plates. Though the collection thus laboriously discussed presented many points of interest, Brady was forced to admit that it was far from representative of what the ocean's resources were likely to contain, and that the last word had not been said as to methods of preserving these organisms. In his Introduction he makes some remarks which bear on a subject previously mentioned:—"The appearance of these minute creatures at the surface depends upon conditions, the nature of which we scarcely at all understand. Night, on the whole, seems to be more favourable than daytime, but even during the day they sometimes appear in numbers so vast as to colour the sea in wide bands for distances of many miles. This appearance has been noticed, perhaps, most frequently in the tropics; but even in the Arctic seas some species, especially *Calanus* (*Cetochilus*) *finmarchicus*, are at times so abundant as to constitute, it is said, a most important item in the food of the whale. So far, indeed, as number and size of individuals are concerned, it would appear that the cold water of the Arctic and Antarctic seas are even more favourable to the growth of Copepoda than the warmer seas of the Tropics."

With his frequent and arduous contributions to scientific literature Brady combined, from 1857 till about 1890, the conscientious exercise of an exacting profession, practising as a doctor in Sunderland, "and after that gave up his time to his professorship at the Armstrong College, until he resigned in 1906 and . . . . came to live in Sheffield." His professorship he had held since 1875. He married in 1859 and had one son and three daughters, losing his wife ten years and his son one year before his own death. Two of his daughters are married to members of his own profession, one to Dr. Charles Atkin of Sheffield and another to Dr. R. S. Hubbersty of Sunderland, the third remaining with her father to the close of his days. He died on Christmas evening, 1921. Till the last year of what he himself described as his long and happy life, he had never realised that he was old. Apart from science his amusements had all been of a tranquil kind—gardening, photography, and the game of bowls. A friend, who had been reading over many of his writings, tells his daughter that: " Dominating all is the intense love he had for nature, religion, and poetry." Another friend, who often walked with him, tells her of the enjoyment derived from the humour, instruction, and high tone of his conversation. A long correspondence is in harmony with these touches of character.

A letter from Sheffield, dated June, 1915, shows him at eighty-three, away from necessary books, reluctant to undertake fresh work of importance, yet unable to be disobliging. He explains that he had declined an invitation to describe the *Ostracoda* and *Copepoda* collected by the Australasian Antarctic Expedition, 1911–1914, under Sir Douglas Mawson, but that the material had nevertheless been sent him, with further pressure. Now, the Scientific Records of that Expedition show that in Series C the fifth volume contains monographs on the *Copepoda*, the *Cladocera*, and *Halocypridæ*, by G. S. Brady. A fine finish!

T. R. R. S.

FRANCIS ARTHUR BAINBRIDGE, 1874–1921.

In the death of Francis Arthur Bainbridge, at the early age of 47 years, Physiology has lost an enthusiastic and successful investigator and a teacher of ability and influence. He was elected to the Fellowship of the Royal Society in 1919, and it was but a few months later that he showed the first definite signs of the ill-health, which culminated in a brief, acute illness, and death on October 27, 1921.

Bainbridge entered the Leys School with a scholarship in 1888, and passed from there in 1893 with an entrance exhibition to Trinity College, Cambridge, of which foundation he subsequently became a major scholar. His student career was on normal lines for a man of his ability and studious habit, but, on finishing the Natural Sciences Tripos, in both parts of which he was placed in the first class, he left Cambridge for St. Bartholomew's Hospital, shaping his course with a view to the practice of medicine. After qualification, he held several minor appointments in physiology and pathology, while waiting for opportunities of advance in the career which he had then chosen; but his natural bent was already obvious, in the devotion of the time which he could spare from his official duties to research in physiology, which he carried on in the physiological laboratory at University College.

In this period he made a series of investigations into the mechanism of lymph-formation, which brought clearly into view the influence of activity in gland cells on the outpouring of lymph from the blood-vessels of the gland. These and other phenomena of lymph-formation, which some had regarded as indicating a process of active secretion by the capillary endothelium, he brought into harmony with Starling's simpler physical conception, producing ingenious experimental evidence in support of that point of view. He became active also in the new field of investigation opened up by Bayliss and Starling's discovery of Secretin, which was made at this time. Bainbridge, however, unable as yet to give to physiology an undivided allegiance, had neither the time nor the impulse to acquire that full command of specialised technique and experience needed for an essentially biochemical investigation. When eventually, in 1905, he abandoned the idea of medical practice, it was to accept the Gordon Lectureship on Pathology at Guy's. Here, with A. P. Beddard, he began a series of elegant experiments on the secretion of urine by the frog's kidney, which he resumed on his return to physiology in later years. With Beddard also he effected a useful revision of then current views as to the meaning of the sequelæ of partial nephrectomy.

Bainbridge left Guy's in 1907, and, wandering for some years yet further from physiology, was responsible, at the Lister Institute, for valuable contributions to the study and classification of the paratyphoid and food-poisoning group of bacilli. This work formed later the basis of his Milroy Lectures to the Royal College of Physicians.

It would seem that, up to this period, Bainbridge had so divided his aims and his interests that onlookers found it difficult to place him. The few who knew him intimately felt that his worth and ability had yet to win a full and general appreciation. When the intermittent manner of its accomplishment is remembered, it appears that his output in physiology was already remarkable; but he had never yet been in a position to regard it as his life-work, and his sound contributions to other branches of medical science seemed, with many, to weaken rather than strengthen his claim and his promise as a physiologist.

It was not till 1911 that his election to the Chair of Physiology of Durham University, in Newcastle, enabled Bainbridge at length to devote himself whole-heartedly to the line of work which most truly held his interest, and for which he felt himself best fitted by early training and natural aptitude. His department soon attained a high standard of efficiency in the training of students, and he resumed, with the late J. A. Menzies, his experiments on the frog's kidney. Later, he entered upon the series of investigations on the adjustment of the heart-beat to the demands of muscular exercise, which will probably rank as his most important and permanent contribution to Science. With Menzies also he wrote what has become one of the most popular and useful of the shorter text-books of physiology for medical students.

When war broke out in 1914 Bainbridge took a commission in the R.A.M.C., and doubled the duties of his Newcastle Chair with those of Medical Officer at a neighbouring military hospital. In 1915 he was appointed to the Chair of Physiology at his old hospital, St. Bartholomew's, in London, and combined the duties of this new Chair with those of an officer in the Anti-gas Service, experimenting at Millbank, or touring the country as a training-officer in defensive measures. Though he was active, and even rather athletic by inclination, his constitution was never really robust, and was not fitted for this unremitting overwork. The growing demands of his teaching necessitated the resignation of his commission, and, during the period of moderate health remaining to him, he found time to complete a monograph on "The Physiology of Muscular Exercise," which was published at the end of 1919, and was received with general appreciation by physiologists and others interested in its subject. It was, indeed, in many ways a model of what such a survey of knowledge should be, and the best proof which Bainbridge has left of one aspect of his ability. The presentation was clear and logical, and it showed a sound instinct for essentials, in a subject of which the main outlines have too often been obscured by controversy concerning details. The note of personal contact with the problem was clearly heard, but not unduly emphasised, and the monograph was generally recognised as a sound and scholarly achievement.

To those who had known Bainbridge long, it seemed that he changed far less than most men do. In later years the circle of his friendship widened greatly, but the associations formed in the early days always had first place in his regard. Similarly, his experience and his influence widened; but those who had the privilege of intimacy with him found that his tastes and convictions, his fundamental attitude to life, changed remarkably little from those of his student-days. It may be that a rather prematurely cautious and reticent habit of mind had hampered his earlier career.

His stature was small, his manner quiet and unimpressive, and he had no great natural gift of vividness or eloquence in public speaking, though he became a clear and effective lecturer to students. These things, with his hesitation in committing himself definitely to the work for which he was best adapted, rather delayed the recognition which was only beginning to come to him in proper measure when he died. With health and opportunity, he would have carried much farther the work that he had begun.

Bainbridge married in 1905, and, to those who knew him well, the thought of his wife's brave and buoyant comradeship, through times of hesitation and disappointment, of success and recognition, and, finally, of stubborn fight against ill-health, is inseparable from his memory. His widow, his sisters, and his young daughter will have, in their sad loss, the sympathy of all who knew him, and especially of that smaller group, who, through years of intimacy, had come to know and to prize the steadfast affection, the quiet but unwavering loyalty to ideals and convictions, which were outstanding features of a fine character.

H. H. D.

AUGUSTUS DÉSIRÉ WALLER, 1856–1922.

It was with the greatest surprise and deepest regret that his numerous friends heard of the sudden death of Prof. Waller a few weeks ago. He was ill for only twelve days; he had a slight stroke, from which his medical attendants thought he would fully recover, but other and severer hæmorrhages followed and he passed quietly away on March 11th.

He was born on July 12th, 1856, so that at his death he was in his sixty-sixth year. He was in full vigour and no one would have expected from his energy, both mental and bodily, that the end would come so soon.

He was the only child of Dr. Augustus Volney Waller, F.R.S., and like his father he became famous in physiology. His father's name in adjectival form is familiar throughout the world, and his discovery of what is now called "Wallerian degeneration" stands out as one of the most important milestones in physiological history. His son was very jealous of the reputation of his father, and I remember one of the few occasions on which I have seen him roused to anger was when he thought his father's work had been misrepresented. He dedicated his 'Introduction to Human Physiology' to his father's memory, summarising the latter's work in the words:—

> Emigration of leucocytes, 1846.
> Degeneration and regeneration of nerve, 1856.
> Cilio-spinal region, 1851.
> Vaso-constrictor action of sympathetic, 1853.

One of his sons still carries on the physiological tradition, Dr. William Waller being one of the junior staff at the University of Liverpool.

The second Waller, whose loss we have now to deplore, was born in Paris, where at the time his father was pursuing his work, and he received his early education at the Collège de Genève. This early training had considerable influence subsequently. He wrote and spoke French fluently, and he usually communicated the results of his research work to learned societies in both countries. Some of his mannerisms, his expressive and eloquent gestures, were doubtless to be traced to the same source.

In 1870 his father died and he went with his mother to Aberdeen, where, after graduating M.B., C.M., in 1878, he finally took his M.D. in 1881. He studied also in Edinburgh, but soon migrated to London, and worked at University College under the then Professor of Physiology, Dr. (afterwards Sir John) Burdon Sanderson. He received grants from the British Medical Association to assist him in his investigations, and in 1884 became Research Scholar under the same body.

His first independent appointment as a teacher was that of Lecturer on Physiology at the London School of Medicine for Women, where he met the lady who became his wife and life-long companion and helper. He then obtained a similar post at St. Mary's Hospital, and finally, about twenty years ago, he was appointed Honorary Director of the Physiological Laboratory at the University of London, with the title of Professor. With characteristic keenness he had explored the buildings at South Kensington in which the University had just been housed, saw the possibilities of using profitably a suite of disused rooms, and, with the help of friends, secured them for the useful purpose to which, through his efforts, they were ultimately applied.

Waller's name became known to the physiologists chiefly through his work on the electrical phenomena of the nervous system and of the heart. In this work his ingenuity in the devising of experiments and apparatus came to the fore. In 1913, he summarised his many years' work on the heart in the Oliver-Sharpey lectures given before the Royal College of Physicians. I cannot do better than quote from a letter by Sir Thomas Lewis, F.R.S.,* the foremost of present day electro-cardiographers, regarding the value of this branch of research. He wrote:—

"May I add a few words of tribute to the memory of Prof. Waller, whose death will be much regretted by both physiologists and physicians in this country and in many other lands. He was a man of unusually keen intellect, and has been for many years a notable figure in British physiology. His brilliant powers of exposition will long render his demonstrations at the Physiological Society memorable. His early work on electro-physiology was extensive, thorough, and is well known. He was the first to show that the currents set up by the beating of the human heart can be recorded; he was the first to obtain a human electro-cardiogram; this has been the main though by no means his sole contribution to the science of experimental medicine. The discovery long preceded the introduction of the string galvanometer, and was the more remarkable in that it was accomplished in the eighties."

The electrical phenomena in other living structures also attracted his attention; he published numerous papers on the currents found in the retina, nerves, muscles, skin, etc., and also in plants. They are summarised in his book "Signs of Life from the Electrical Aspect," published in 1903. The high estimation in which his work was held was shown by his election as F.R.S. at the comparatively early age of 36, in 1892.

During his investigation of nerve and muscle, he made observations on the effects of anæsthetic vapours and gases on their electrical responses, and thus he became interested in clinical anæsthesia, and in lectures, demonstrations and discussions insisted on the necessity of accurate dosage in the administration of these dangerous means of alleviating suffering, especially in reference to chloroform. He invented an apparatus for controlling the percentage of the anæsthetic in the air a patient breathed, for he was convinced that deaths under chloroform could be prevented with proper care. In 1901, a Committee was established by the British Medical Association to go thoroughly into the matter, and Waller became one of its most earnest members, and was at one time its Chairman. The report which was published ten years later is a most valuable document, and formed another example of how Waller's academic work was fruitful from the practical standpoint.

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* 'Brit. Med. Journ.,' 1922, vol. i, p. 459.

During the last few years of his life, he became interested in three new subjects, and threw himself with his usual enthusiasm into all of them. His laboratory at one time was, or seemed to be, wholly devoted to one of these; at another time, it was one or other of the remaining two. This was not only the case at the South Kensington laboratory, but the same fervour was manifested at his private but well known laboratory at his home in Grove End Road. The old roomy studio (for the house formerly belonged to a well-known artist) was transformed not only into a laboratory, but became the principal living room of the professor and his family, where they received their friends scientific and otherwise. It opened into a spacious garden which also was a great recreation to the workers. The large table in the middle of the room was crowded with electrical and other apparatus, when by an ingenious arrangement of pulleys, the top was suddenly hoisted ceiling-wards, and a full-sized billiard table was revealed. Waller was as keen on games as he was on work, and billiards were not the least of his accomplishments. When he first took to driving a motor car, all his energy seemed devoted to mastering the intricacies of its mechanism and management. Another of the many other interests of his many-sided life was his fondness for animals, and especially for bull-dogs. The ancestor of several generations of these was Jimmy who became well known as his constant companion in the car and in the laboratory. He was the faithful guardian of the car when his master left it standing, his fierce countenance being sufficient to repel intruders in spite of his gentle nature. Jimmy appeared at several Royal Society Soirées, with his paws in basins of salt solution which were connected to a galvanometer or electrometer in order to demonstrate the accompanying electrical changes of his heart's activity to an admiring audience. The Home Secretary of that day, Mr. Herbert, now Lord Gladstone, had to explain to anti-vivisectionist members of the House of Commons that this was not a brutal experiment, and that Jimmy suffered as much or as little pain as a child paddling in the sea.

But these are digressions. I had begun to speak of the subjects which interested Waller in later years. One of these which only needs a passing mention was of a polemical nature, and related to certain movements which with the aid of high magnification can be shown to occur in plants. Waller attributed these not to growth, but to mere turgescence such as occurs when many substances are placed in water.

The other two topics to which he devoted himself were of a more serious nature. One of these was the investigation of the so-called "emotive response," and the other the measurement of the cost of muscular work by estimation of the carbonic acid exhaled.

Both were pursued with characteristic intensity; his friends inveigled into the laboratory had to submit to be put "on the wires" in order that the change in the resistance of their skin which occurs under various emotions might be measured and recorded; one of the many interesting outcomes of this work was that most people reacted to the threat of an injury, such as a burn, much more strongly than to actual pain. During the air raids he had his wife and others "on the wires" and noticed a corresponding effect. The investigations on muscular work were carried out on himself, his friends, soldiers, colliers, bootmakers, printers and many other classes. He found the severity of the work and the output of CO_2 were parallel. His method has been criticised, as no account was taken of the oxygen usage, but Waller never claimed absolute accuracy; he regarded his "short method" for testing the cost of work as a practical means to an end which can be accomplished only with accuracy by much longer and more complicated methods. It can be carried out while the work is in actual progress. It was his intention to have compared the two methods in a parallel series of experiments had his life been spared, but in criticising his critics showed that their longer methods did not always give better results than his own.

Waller's contributions to the literature of his subject were numerous, and in addition to the books already mentioned, they were mainly published in the 'Proceedings' and 'Transactions' of our Society, and in the 'Journal of Physiology.' His academic distinctions were also numerous. In 1889 he was made a Lauréat of the Institute of France; he received the Aldini prize from the Royal Academy of Science of the Institute of Bologna. He was a corresponding member of many foreign learned societies and academies, and an honorary member of the Council of the University of Tomsk.

Prof. Waller married Alice Mary, daughter of the late Mr. George Palmer, M.P. for Reading. It was an ideal union. Mrs. Waller shared in all his work, and he was a devoted husband; her recent illness caused him to relinquish his other work; he took a room near the nursing home where she was in order to be with her constantly; later, when she returned home he used to carry her to and from her room; his anxiety about her was most intense.

He was an equally devoted father; he had three sons, and two daughters, all of whom survive him except the youngest daughter, whose tragic death from drowning seemed to leave a permanent mark of sorrow in his character. He was no doubt a physiologist first, but in this imperfect survey of his life's work I have endeavoured to show that there were other sides to his personality. He passed through many turmoils, of which the last (the attempt to close the University laboratory while he was in full vigour and it was in full swing of useful work) was by no means the least. He won that victory; he was a good fighter, an ardent and affectionate friend and a great man.

W. D. H.

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